# Nonclinical Study on Allergen-Specific Sublingual Immunotherapy with Allergen Extract of Japanese Cedar Pollen

# Nonclinical Study on Allergen-Specific Sublingual Immunotherapy with Allergen Extract of Japanese Cedar Pollen

A Dissertation Submitted to

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# Abbreviations

| | |
|---|---|
| **2AA** | 2-aminoanthracene |
| **ADME** | Absorption, Distribution, Metabolism and Excretion |
| **AF-2** | 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide |
| **AIT** | Allergen specific immunotherapy |
| **APC** | Allergen-presenting cells |
| **CHL** | Chinese hamster lung |
| **DMN** | Dimethylnitrosamin |
| **EAACI** | European Academy of Allergy and Clinical Immunology |
| **ELISA** | Enzyme-Linked Immunosorbent Assay |
| **GI** | Gastrointestinal |
| **GLP** | Good Laboratory Practice |
| **HE** | Hematoxylin and eosin |
| **HRP** | Horseradish peroxidase |
| **JAU** | Japanese allergy units |
| **JC** | Japanese cedar |
| **JCP** | Japanese cedar pollen |

| | |
|---|---|
| **JSA** | Japanese Society of Allergology |
| **MMC** | Mitomycin C |
| **MNPCE** | Micronucleated polychromatic erythrocyte |
| **NDA** | New drug application |
| **NOAEL** | No-observed-adverse-effect level |
| **OD** | Optical density |
| **PD** | Pharmacodynamics |
| **PK** | Pharmacokinetics |
| **PCA** | Passive cutaneous anaphylaxis |
| **RET** | Reticulocyte |
| **SCIT** | Subcutaneous immunotherapy |
| **SLIT** | Sublingual immunotherapy |
| **SLS** | Sodium lauryl sulphate |
| **SPF** | Specific pathogen-free |
| **Th1** | T-helper type 1 cell |
| **Th2** | T-helper type 2 cell |
| **Treg cell** | T-regulatory cell |

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# Chapter I. PREFACE

## 1 Japanese Cedar Pollinosis

Seasonal allergic rhinitis, also named Japanese cedar (JC) pollinosis, is caused by Japanese cedar pollen (JCP). JC pollinosis is a type-I allergic disease and is the most common disease in Japan and thus is considered a national affliction (Kaneko et al., 2005; Yamada et al., 2014). More than one third of all Japanese population have JC pollinosis, and this number has significantly increased in the last 2 decades (Okubo et al., 2011; Yamada et al., 2014).

The main symptoms are sneezing, watery rhinorrhea, nasal blockage and itching of eyes which are caused by chemical modulator release such as histamine from mast cells and basophils following to the crosslinking of the receptors by allergen-IgE binding. In addition, many JC pollinosis patients have also been sensitized to cypress pollen scattered after the JCP season by IgE cross-reactivity between JCP and cypress (Di Felice et al., 2001), which causes a persistent long-term symptom onset.

## 2 Allergen Specific Immunotherapy

Allergen specific immunotherapy (AIT) was introduced to treat pollinosis or allergic rhinitis more than 100 years ago (Noon, 1911; Freeman, 1911; Freeman, 1930). Since then, AIT has been used to treat allergic diseases caused by inhalant allergens and is an effective treatment for patients with seasonal or perennial allergic rhinoconjunctivitis and asthma. AIT is the practice of administering gradually increasing quantities of an allergen extract to an allergic

subject (Figure 1) to ameliorate the symptoms associated with the subsequent exposure to the causative allergen (Mailling and Weeke, 1993; Bousquet et al., 1998; Cappella and Durham, 2012; Eifan et al., 2013; Canonica et al., 2014). AIT is currently considered to be curative treatment with allergic disease although symptomatic treatment such as antihistamines and nasal corticosteroids allows tentative relief from symptoms (Bousquet et al., 1998; Canonica et al., 2014).

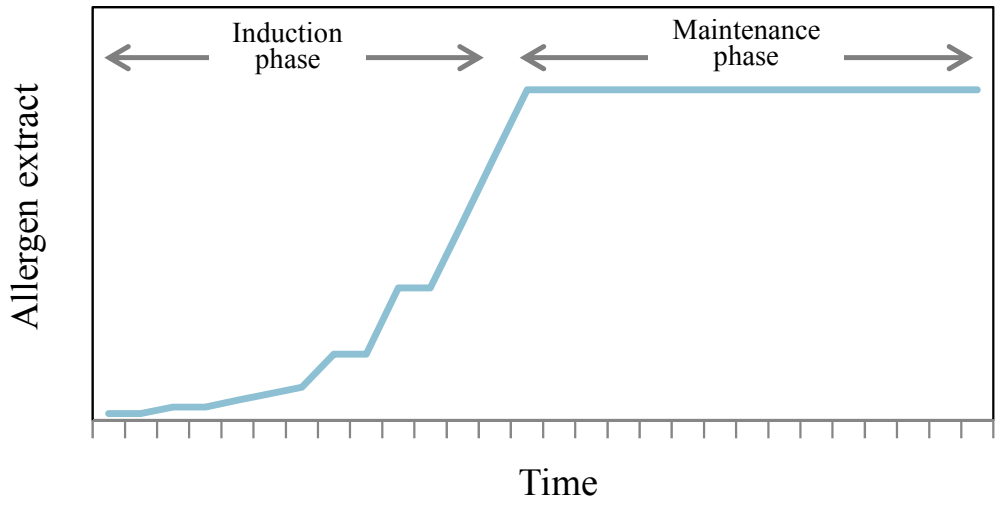



**Figure 1. Gradually increasing quantities of an allergen extract to an allergic subject in allergen specific immunotherapy**

Subcutaneous immunotherapy (SCIT) has been introduced since 1960's at Japan, and it was used to treat JC pollinosis (Okubo et al., 2011). However, it is absolutely disadvantage with a limited usage of SCIT because of severe systemic adverse effects such as anaphylaxis (Bernstein et al., 2004) and inconveniences such as injection site pain and frequent hospital visits known as general drawbacks of SCIT. To solve such adverse effects and inconveniences of SCIT, several alternative noninvasive allergen application strategies have

been investigated, using the oral mucosal, intranasal, bronchial or oral route (Valovirta et al., 2004). Only the administration of allergens via the oral mucosal route (allergen-specific sublingual immunotherapy: SLIT) gained increasing significance as an alternative application strategy to classical subcutaneous injections. SLIT is strongly considered to be a safe and efficient treatment for respiratory allergy, and has been introduced as an alternative to SCIT (Didier et al., 2011; Nelson et al., 2011; Cox et al., 2012; Durham et al., 2012). In addition, SLIT reduces the burden on patients compared with SCIT because it can be administered at home and is associated with fewer severe side effects. In clinical use of allergen extract for SLIT, the allergen extract is placed under the tongue for 1 or 2 minutes and then swallowed (SLIT-swallow) or spat out (SLIT-spit) (Canonica et al., 2014). Currently, SLIT-swallow is performed followed the basis of clinical results and pharmacokinetic considerations (Canonica and Passalacquea, 2003).

## 3 Standardized Allergen Extract

The allergen extract is indispensable for both of diagnosis and treatment for allergy disease. The use of standardization of allergen extract is considered to be essential to raise reliability in the diagnosis, efficacy and safety in the treatment (Reed et al, 1989; Platts-Mills and Chapman, 1991; Ito, 1992) because quality of the extract obtained from native materials is affected by various reasons. Several position statements were issued from American Academy of Allergy and Immunology (1992 and 1993) and American Academy of Allergy, Asthma and Immunology (1997) on the recommendation of use of standardized extracts for the diagnosis and treatment for allergic diseases. The standardization

of allergen extract is the most important to keep their quality as well as batch-to-batch control among products from manufacturers.

In Japan, the Japanese Society of Allergology is the only authority to conduct standardization of allergen extract. In fact, allergen extract of JCP (henceforth described as JCP-allergen extract) was a first extract standardized by the Japanese Society of Allergology in 1995. A standardized JCP-allergen extract has been used for SCIT treatment of the JC pollinosis patients from 2000 (Torii Pharmaceutical Co. Ltd., Pharmaceutical interview form; Standardized allergen extract for SCIT [TORII], Japanese Cedar Pollen). The potency of JCP-allergen extract is expressed as Japanese allergy units (JAU) as defined by the Japanese Society of Allergology based on skin tests of allergy patients. A liquid containing 200 or 2,000 JAU/mL is formulated by dilution of the standardized JCP-allergen extract original solution (10,000 JAU/mL; Torii Pharmaceutical Co. Ltd., Tokyo, Japan) containing the major allergen Cry j 1 (Yasueda et al., 1983) at 7.3–21.0 μg/mL (Yasueda et al., 1996) to provide the indicated potency.

## 4 Sublingual Immunotherapy Droplet of Japanese Cedar Pollen

Several clinical studies in small patient populations have shown that SLIT might be effective for the treatment of patients with JC pollinosis (Horiguchi et al., 2008; Okubo et al., 2008; Fujimura et al., 2011). In these clinical investigations, SCIT products were used for sublingual administration (SLIT) as an alternative route of administration. For the application to SLIT, a modified liquid formulation of the SCIT product of standardized allergen extract of JCP has been developed (Torii Pharmaceutical Co. Ltd, Tokyo, Japan) (Table 1).

**Table 1. Sublingual droplet of Japanese cedar pollen**

| Potency of allergen | Use in SLIT to JC pollinosis | Cry j 1 contents |
|---|---|---|
| 10,000 JAU/mL | Original solution | 7.3-21 μg/mL |
| 2,000 JAU/mL | Induction phase<br>Maintenance phase | Approximately<br>3 μg/mL |
| 200 JAU/mL | Induction phase | Approximately<br>0.3 μg/mL |

Doses of 200 and 2,000 JAU/mL were used in SLIT. The solution of 200 and 2,000 JAU/mL were formulated by dilution using original solution of standardized allergen extract of JCP 10,000 JAU/mL. A container with a pump to provide 0.2 mL in one push was used in the induction phase at doses of 200 and 2,000 JAU/mL. A single usage pouch for the maintenance phase was used to deliver 1 mL of 2,000 JAU/mL.

## 5 Nonclinical Study in Drug Development Process

In drug development, nonclinical studies (pre-clinical studies) are essential process before initiating clinical trials for assessment of both safety profile and biological activity (Figure 2). The main goal of nonclinical studies is to determine the ultimate safety profile of new drug. In fact, different types of approach on nonclinical studies are required by authority following a new drug application (NDA) process for each drug.

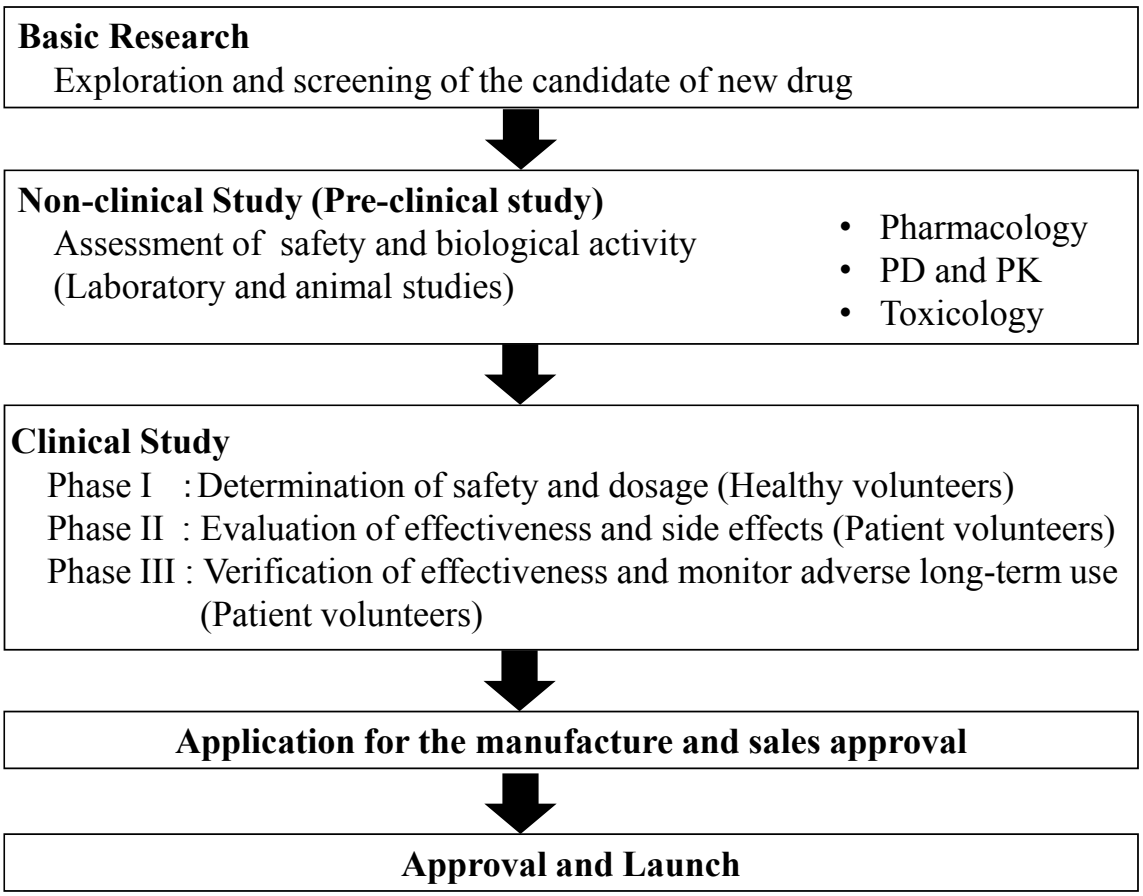



**Figure 2. Overview of drug development process.**

PD: Pharmacodynamics, PK: Pharmacokinetics

For instance, the profiles of pharmacology, pharmacodynamics (PD), pharmacokinetics (PK) and toxicology are examined in *in vitro* and *in vivo* studies using animals. These data sets allow estimating a range of safe dose of the drug which is suggested to use in clinical trials, mainly Phase I. In Phase II of clinical trial, the treatment dose need to be defined either results of Phase I study as well as PK/PD data in animals. To guarantee the quality of data in nonclinical studies, the toxicological studies for NDA should be conducted following Good Laboratory Practices (GLP) compliance according to the test system guideline.

In Japan, there is no specific regulatory guideline for AIT drugs in Japanese NDA process. Thereby, nonclinical data in AIT products should be applied to the regulatory requirements as well as a small molecule of drug. Here, nonclinical studies were conducted as a pre-clinical stage for the new drug development of SLIT product which is modified liquid formulation of the SCIT product. Biological profile and the safety profile of standardized JCP-allergen extract were investigated. Furthermore, the concepts of another nonclinical study (i.e., pharmacology and ADME) were discussed regarding the current position in the new drug development of allergen immunotherapy drug.

(1) Pharmacological profile of JCP-allergen extract

AIT has been well known as an effective treatment with type-I allergic patients as well as only one therapy to acquire to inducing tolerance against specific allergen (Jutel et al., 2006; Eifan et al., 2013; Canonica et al., 2014). The mechanism of action (tolerance induction) by repeated allergen administration is not clear entirely. To investigate mechanism of action on

AIT, the disease model using animals is a key to develop a new drug. In the reports, a few animal models using sensitization of JCP has been introduced and tried to mimic pathophysiology of allergic conditions (Tsunematsu et al., 2007). However, it is not fully established and validated as a disease model.

For understanding the efficacy and mechanism of action by the allergen extract in AIT, two different aspects of the extracts are existed; antigen profile in immunological response and allergen profile in allergenic response (Figure 3, refer to **introduction of Chapter II**). The antigen profile in repeated administration is discussed **section 4 of Chapter II**. For evaluating the efficacy in terms of allergen potency, the allergen-IgE binding is a very important which is followed by release of chemical modulators such as histamine from mast cells. The standardized JCP-allergen extract including Cry j 1 and Cry j 2 allergen proteins (Figure 5, **introduction of Chapter II**) known as JCP major allergens (Yasueda et al., 1983; Sakaguchi et al., 1990) have a binding potency to specific IgE in sera of JCP-allergic patients (Figure 6, refer to **introduction of Chapter II**). It indicates that JCP-allergen extract elicits a total allergic potency. Allergic specific response was examined in rat (refer to **section 2 in Chapter II)**.

(2) Pharmacodynamics and Pharmacokinetics (ADME) of JCP-allergen extract

In the process of development of a new drug with small molecule, pharmacokinetics in absorption, distribution, metabolism and excretion are investigated by *in vitro* and *in vivo* assay. Generally, the candidates of drug (small molecule) are possible to be traced after dosing in the body using labelled candidate. However it is difficult to fully investigate regarding ADME on allergen extract because main factors to induce efficacy are

proteins which are usually metabolized by enzyme in the body fluids as generally known.

As a characteristic mechanism of SLIT, it is mainly considered to have important role in lymph node where is very close to administration site such as a mandibular lymph node. Taken this common understanding, the tissue concentration and distribution of [$^{125}$I] Cry j 1 (Cry j 1; major allergen of JCP) were examined (Ohashi-Doi et al., 2011). The stability of allergen protein in saliva and stomach juice was discussed in **section 3 of Chapter II**.

(3) Toxicology

The purpose of the toxicological studies conducted here is to estimate physical property of JCP-allergen extract to induce any toxic effect by repeated dosing. It provides safety profile of JCP-allergen extract. The study design is shown in Table 2.

**Table 2. Study design and implementation on safety evaluation studies of JCP-allergen extract**

| Study | Species of animals | Number of animals | Route | Test article | Dosage |
|---|---|---|---|---|---|
| ***Repeated dosed toxicity (Chapter III)*** | | | | | |
| 26 weeks repeated | Rats (male and female) | 12 | p.o. | Saline | 0 JAU/kg |
| | | | | Glycerin solution | 0 JAU/kg |
| | | | | 200 JAU solution | 200 JAU/kg |
| | | | | 2,000 JAU solution | 2,000 JAU/kg |
| | | | | 10,000 JAU solution | 10,000 JAU/kg |
| ***Local irritation (Chapter IV)*** | | | | | |
| 7 days repeated | Rabbits (male) | 3 | SL | Saline | 0 JAU/site |
| | | | | Glycerin solution | 0 JAU/site |
| | | | | 2,000 JAU solution | 400 JAU/site |
| 14 days repeated | Rats (male) | 3 | p.o. | Glycerin solution | 0 JAU/site |
| | | | | 10,000 JAU solution | 250 JAU/site |
| | | | | | 1,250 JAU/site |
| | | | | | 6,250 JAU/site |
| ***Genotoxicity (Chapter V)*** | | | | | |
| Ames test | - | - | - | Distilled water | 0 JAU/plate |
| | | | | Glycerin solution | 0 JAU/plate |
| | | | | 10,000 JAU solution | 31.25–1,000 JAU/plate |
| Chromosomal aberration test | - | - | - | Distilled water | 0 JAU/mL |
| | | | | 10,000 JAU solution | 625–2,500 JAU/mL |
| *In vivo* micronucleus test | Rats (male) | 5 | s.c. | Distilled water | 0 JAU/kg |
| | | | | Glycerin solution | 0 JAU/kg |
| | | | | 10,000 JAU solution | 12,500 JAU/kg |
| | | | | | 25,000 JAU/kg |
| | | | | | 50,000 JAU/kg |

SL: Sublingual
200 JAU solution: Standardized JCP-allergen extract with allergen potency of 200 JAU/mL
2,000 JAU solution: Standardized JCP-allergen extract with allergen potency of 2,000 JAU/mL
10,000 JAU solution: Standardized JCP-allergen extract with allergen potency of 10,000 JAU/mL

Treatment groups are compared with vehicle control group (0 JAU/plate and 0 JAU/kg). Vehicle group (0 JAU/plate and 0 JAU/kg) are compared with control group; distilled water for Ames test, chromosomal aberration test and *in vivo* micronucleus test; saline for repeated toxicology and local irritation.
JAU, Japanese allergy units; s.c., sub-cutaneous; p.o., per oral; PBS, phosphate buffered saline.

## 6 Construction of This Article

In **Chapter II**, biological profile of standardized JCP-allergen extract is focused based on different two aspects of antigen profile and allergen profile. JCP-allergen extract possess both potency of antigen profile and allergen profile, and it is related to the mechanism of immunotherapy of JCP-allergen extract and also itself is causative material for JC pollinosis. In **Chapter III**, safety evaluation on systemic toxicity of repeated dosing is focused. Repeated 26-week oral toxicity study in rats was conducted to evaluate the effect of daily long time treatment of standardized JCP-allergen extract intended to be used for daily treatment by sublingual administration for several years. In **Chapter IV**, local irritation is focused on oral mucosa as first contact site and gastrointestinal (GI) tract mucosa as second contact site with consideration of route of SLIT product. In **Chapter V**, safety evaluation on genotoxicity of standardized JCP-allergen extract is focused by *in vitro* and *in vivo* genotoxicity studies. At last, conclusion and prospective in this article is mentioned in **Chapter VI**.

# Chapter II. BIOLOGICAL PROFILE OF ALLERGEN EXTRACT OF JAPANESE CEDAR POLLEN

## 1 Introduction

It has already demonstrated that AIT has been shown to be clinically effective in patients with allergic rhinitis and/or asthma. The mechanism of allergen immunotherapy is not fully clear, but it is potentially considered that modulation of immuno-response involved in induction of T cells, B cells and T regulatory (Treg) cells specially (Figure 3).

In conventional SLIT, generally high-dose allergen should to be treated due to initiating immune deviation of Th2 responses in favor of a Th0/Th1 response and in the generation of IL-10– and TGF-β–producing $CD4^{+}CD25^{+}$ T cells, possibly Treg cells (Robinson et al, 2004; Novak, 2011; Eifan et al, 2013). In addition, it is founded that induction of increased Treg cells in the nasal mucosa (Radulovic et al., 2008) and sublingual mucosa (Scadding et al, 2010) after AIT, strongly suggesting a potential role for Treg cells in the induction of allergen-specific tolerance in human.

It has also considered that antibody response is a key role in immuno-modulation during AIT (Figure 4) (Akdis and Akdis, 2011). In fact, production allergen-specific IgE and IgG has been investigated in many clinical trials. It was reported that a significant increase in serum-specific IgG4 and IgA was founded after AIT with grass pollen (Francis et al., 2008; Blaiss et al., 2011),

which were associated with an increase in local and/or systemic IL-10 and TGF-β (Nouri-Aria et al., 2004; Pilette et al., 2007; Meiler et al, 2008).

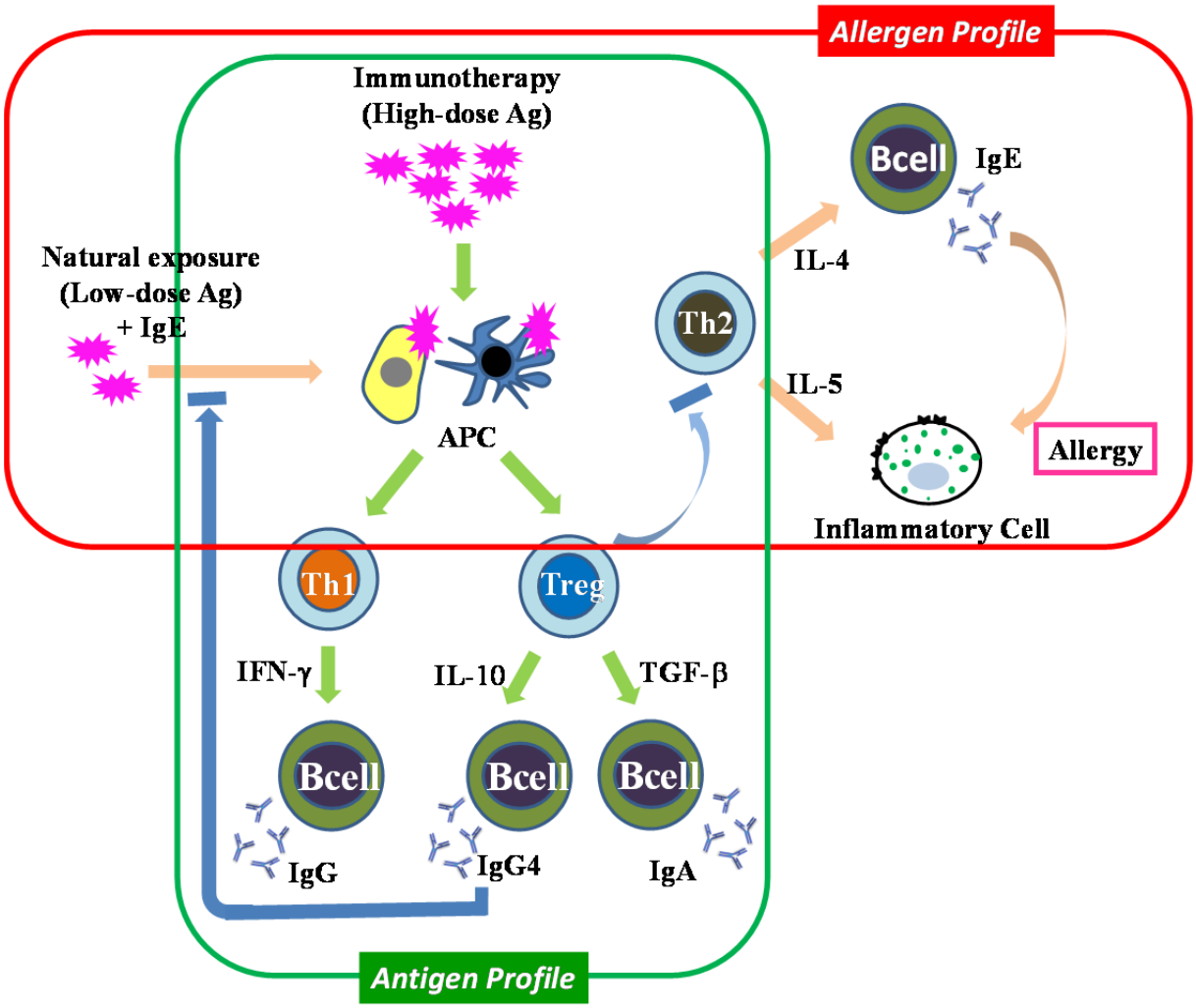



**Figure 3. Immunological mechanism of allergen specific immunotherapy.**

APC: Allergen-presenting cells; Ag: Antigen, IFN-γ: Interferon gamma; TGF-β: Transforming growth factor-beta: Th: T-helper cell; T-reg: T-regulatory cell.

Inflammatory cell indicates mast cell, basophil and neutrophil.

Orange arrows represent immune response pathway to natural exposure (low-dose Ag and IgE) ; green arrows represent immune response pathway to immunotherapy (high-dose Ag); blue blocked lines represent inhibition (high-dose Ag).

Allergen profile: Allergic response induced by IgE-antigen binding followed by degranulation of histamine or other chemical modulator.

Antigen profile: Immunological response by production of immunoglobulin such as IgG, IgA.

Currently, serum levels of allergen-specific IgE and IgG is nominated as candidate biomarkers, although there is considerable debate as to whether these parameters are related to clinical efficacy. In contrast to SCIT, SLIT appears to elicit mucosal IgA responses, which may contribute significantly to tolerance induction (Anthony, 2008).

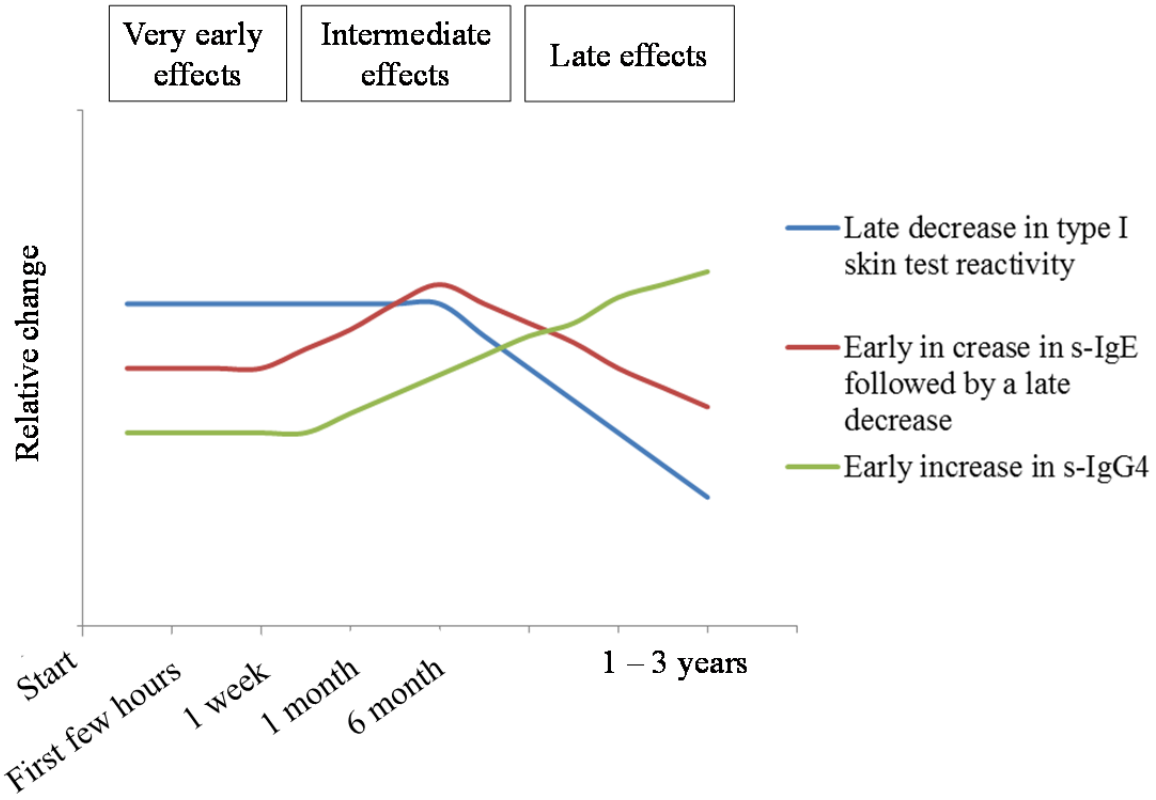



**Figure 4. Immunological changes in allergen-specific immunotherapy.** (Modified from Akdis and Akdis, 2011)

s-IgE: specific-IgE, s-IgG4: specific-IgG4

A part of concept on immunological changes in allergen-specific immunotherapy is indicated although there is significant variation between subjects and protocols. An early increase and a very late decrease in specific IgE level are observed. In particular, the IgG4 level shows a relatively early increase that is dose dependent. In some studies allergen-specific allergen-specific IgG1 and IgA levels also increase. A significant decrease in the allergen-specific IgE/IgG4 ratio occurs after several months. A significant decrease in type I skin test reactivity is also observed relatively late in the course of immunotherapy. These effects are partially demonstrated in SLIT and are rather weak compared with those seen in SCIT.

SLIT has a better safety profile compared with SCIT. This is possibly because oral antigen-presenting cells (APC; mostly Langerhans and myeloid dendritic cells) exhibit a tolerogenic phenotype, despite constant exposure to danger signals from food and microbes. This reduces the induction of pro-inflammatory immune responses leading to systemic allergic reactions (Calderon et al., 2012). Oral tissues contain relatively few mast cells and eosinophils, in comparison with subcutaneous tissue, are less likely to give rise to anaphylactic reactions.

For AIT products, the allergen or/and protein profile should be evaluated due to guarantee induction of immuno-response in the human body. In the case of JCP-allergen extract, major allergen, Cry j 1 and Cry 2 were identified, confirming allergen/protein profile in standardized JCP-allergen extract (Figure 5).

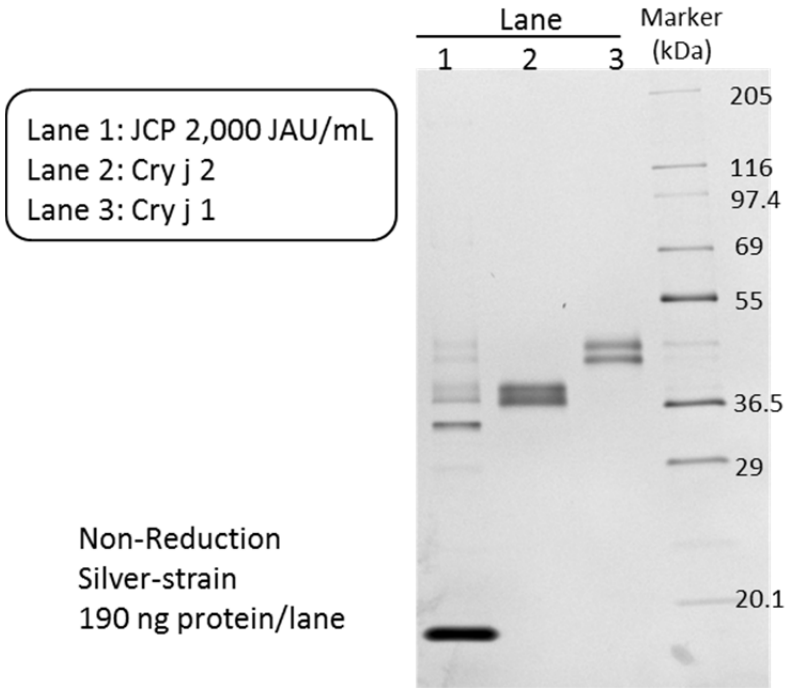



**Figure 5. SDS-PAGE of standardized JCP-allergen extract (2,000 JAU/mL)**

JCP-allergen extract was assessed by SDS-PAGE analysis using a Mini-Protean TGX Precast Gel (4-20% gradient, BIO-RAD) under non-reduction conditions followed by staining with silver.

In addition, the IgE binding activity is recognized as a part of allergic activity because it is known that biding between specific IgE and allergen triggers to induce allergenic reaction. Therefore, it was demonstrated to having potency to binding to specific IgE in sera of JCP sensitized patients with dose dependent manner (Figure 6).

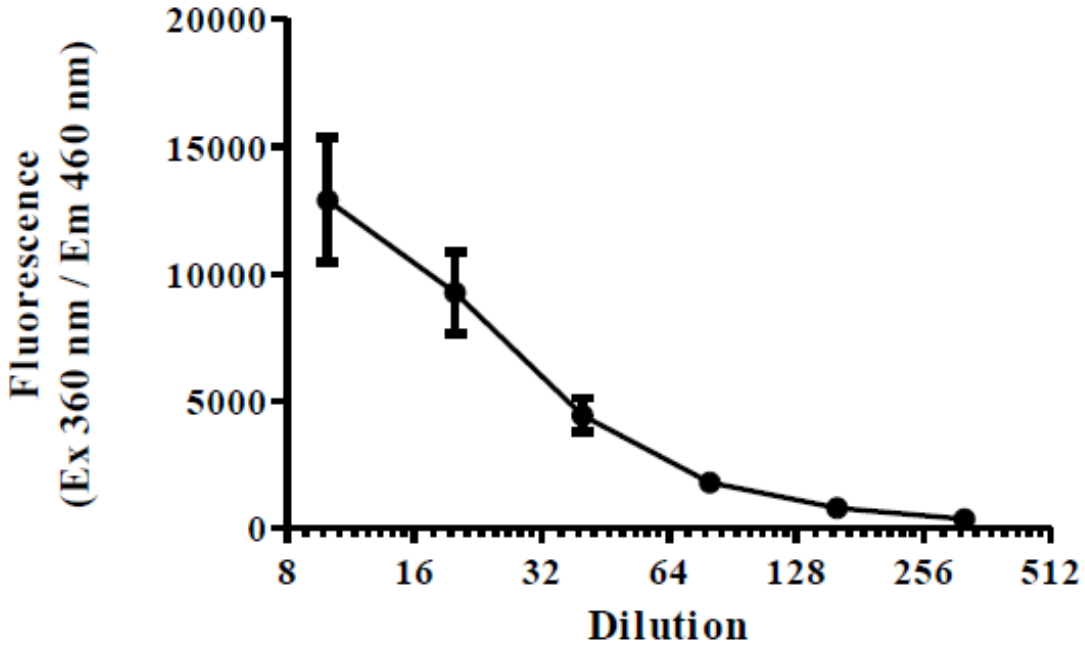



**Figure 6. Total allergic potency of JCP-allergen extract to sera IgE of JCP allergic patients.**

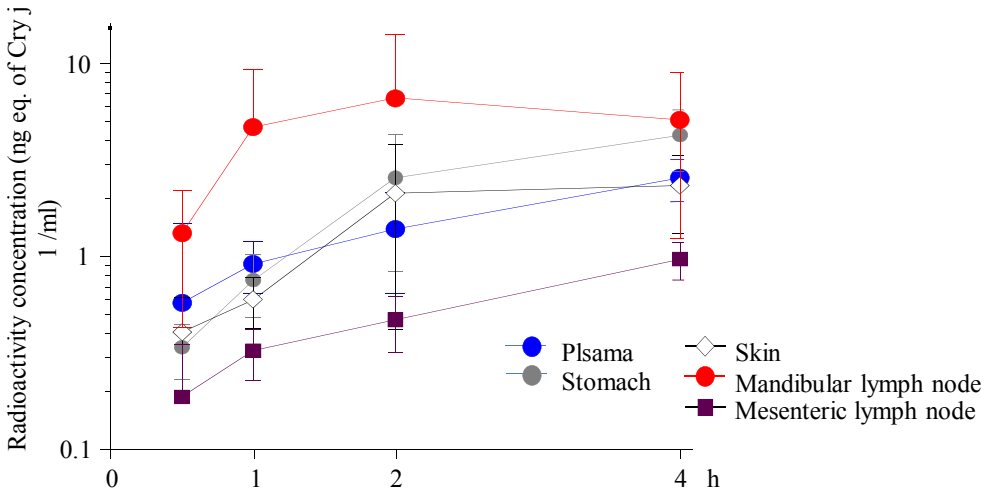
Binding potency to specific IgE in sera of JCP-allergic patients was evaluated by ELISA system. Diluted JCP-allergen extract was incubated with pooled sera from JCP-allergic patients volunteers. The specific IgE binding was detected by anti-human IgE antibody. Values are expressed as fluorescence intensity (excitation 360 nm/emission 460 nm).

To investigate on PK (i.e., ADME), the tissue concentration and distribution of [$^{125}$I] Cry j 1 (Cry j 1; major allergen of JCP) were examined in rats following sublingual or subcutaneous dosing (Ohashi-Doi et al., 2011). As a characteristic mechanism of SLIT, it is demonstrated lower radioactivity in

SLIT-rat sera than SCIT-rat, relative high radio activity in mandibular lymph node located near administrated site (Figure 7) suggesting involved in the local action by SLIT.

In Chapter II, immunological and biological character of JCP-allergen extract is focused; allergen profile by induction of specific allergenic response (PCA) in rat, biological stability of major allergens in saliva and gastric juice, immunological response by repeated administration.

## A) Radioactivity concentration in tissue




## B) Whole Body Radioautography

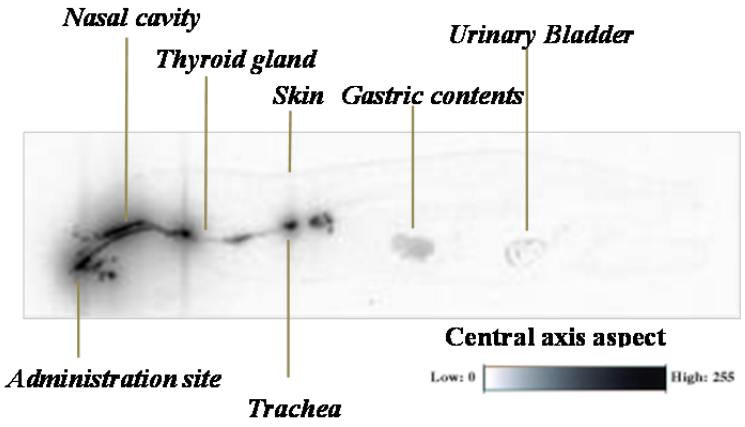



**Figure 7. Radioactivity at 2h after sublingual administration of [$^{125}$I]Cry j 1**

After sublingual administration of [$^{125}$I]-Cry j 1 at a dose of 7.5 µg/animal (male Sprague-Dawley (SD) rats, 7 weeks old), the animals were sacrificed at specified at 2 h (n=3). The designated tissues were collected, and the radioactivity concentrations were measured by the gamma counter (A). Separate group of naïve rats administered by [$^{125}$I]-Cry j 1 were used (n=1). The rats were euthanized with $CO_2$ inhalation at 2 h after single administration. Animals were occluded by 4% carboxymethylcellulose-Na solution, and then body was frozen in a dry ice-acetone mixture and 30 mm-thickness sections were prepared by the cryomicrotome. The sections were covered with a protective membrane and placed in imaging plates (TYPE BAS RS2040, Fujifilm, Japan). The imaging plates were analyzed by a Bioimaging analyser (B).

## 2 Allergic Response in Rat Passive Cutaneous Anaphylaxis Reaction

### 2.1 Introduction

PCA, passive cutaneous anaphylaxis reaction, is typical reaction of immediate hypersensitivity. Sensitized animal is developed by intradermal administration of immunoglobulin and induced cutaneous anaphylaxis reaction by antigen administration. In this study, rats were sensitized with mice sera obtained by immunization of JCP-allergen extract, followed by injection of JCP-allergen extract with trypan-blue. The induced blueing reaction is indication of degranulation of histamine or other chemical modulator induced in the allergic response.

All procedures employed in the experiments were approved by the Animal Ethics Committee of Nihon Bioresearch Center, Gifu, Japan, in accordance with laboratory animal welfare guidelines.

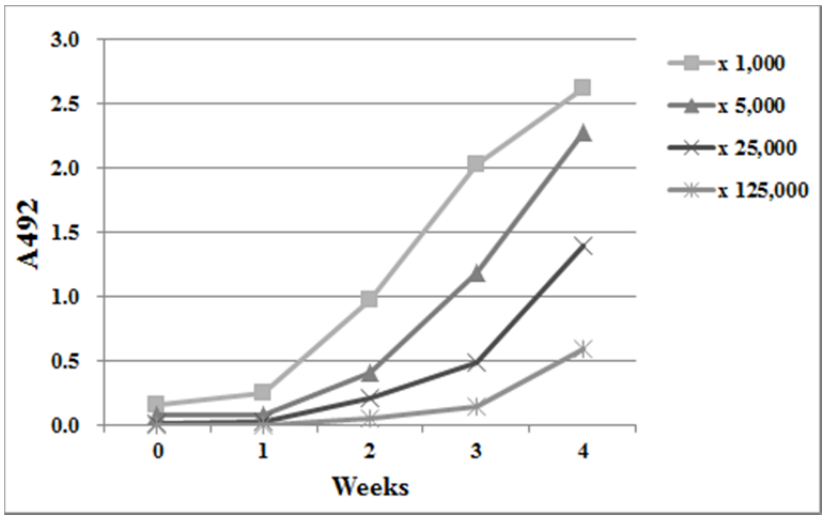
### 2.2 Materials and Methods

Two male Crl:CD (Sprague-Dawley) rats were used (9 weeks old, Charles River, Kanagawa, Japan). Serial dilutions of mouse anti-JCP-allergen extract sera were intradermally injected into shaved dorsal skin. The PCA reaction was measured after a 48-h sensitization period followed by intravenous administration of 10 mg (protein content) standardized JCP-allergen extract original solution (10,000 JAU/mL) in 1 mL/kg of 0.5% Evans blue. After 30 min, the diameter of blueing reaction in the dorsal skin area was expressed as a mean value (mm) of the major and minor diameters in two animals. The PCA titer was expressed by the reciprocal of the final dilution giving a positive (> 5 mm) blueing reaction.

## 2.3 Results and Discussion

The allergen profile of JCP-allergen extract was confirmed by measuring immune responses in animals. Levels of the JCP specific-antibody in serum was significantly increased following intraperitoneal (i.p.) administration with alum compared with naïve serum in mice and rats (Figure 8).

A) Mouse serum




B) Rat serum

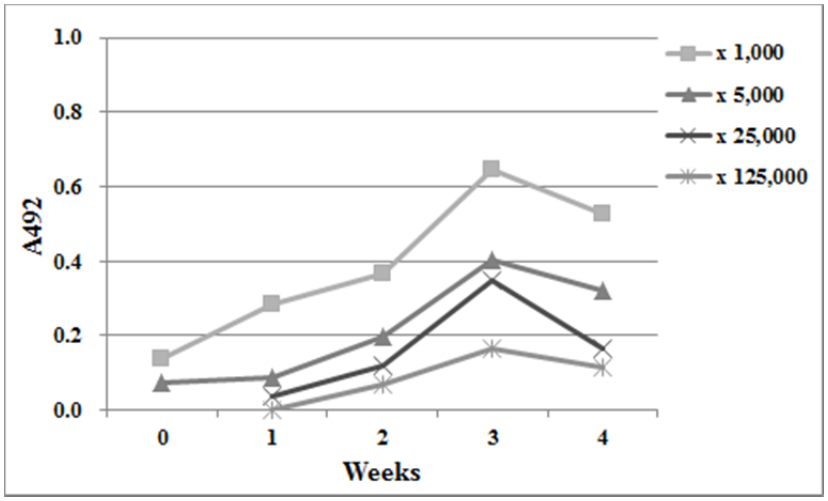



**Figure 8. Induction of immunoglobulin in mice and rats by immunization of JCP-allergen extract.**

BALB/C AnNCrlCrLj male mice (A) and BN/CrlCrlj male rats (B) were administered intraperitoneally JCP-allergen extract with alum at 0, 1, 2, 3 weeks. Serum was analysed for antigen-specific titer by ELISA with indicated dilution ratio.

Next, allergic specific reactions were evaluated by PCA in rats (Figure 9) using mice sera obtained by JCP-immunization (Figure 8). PCA titer was determined to be 128.

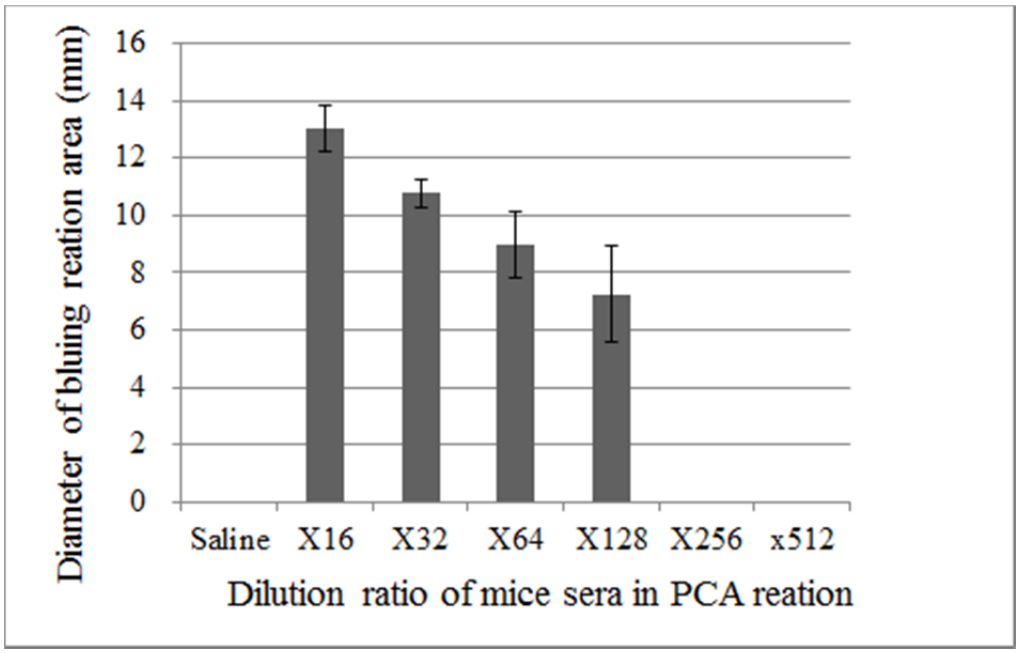



**Figure 9. PCA reaction by JCP-allergen extract in rats.**

Rats were injected intradermally with serial dilutions of mouse anti-JCP allergen extract serum. After 48 h, PCA reactions were elicited by intravenous injection of JCP-allergen extract. Values are expressed as mean ± SD of 4 sites in two animals.

## 2.4 Short Summary

The immune reactions induced by JCP-allergen extract were confirmed by PCA reaction in sensitized-model rats.

## 3 Biological stability of Major Allergens in Saliva and Gastric Juice

### 3.1 Introduction

In SLIT to allergic patients, the allergen extract is placed under the tongue for 1 or 2 minutes and then swallowed (SLIT-swallow) (Canonica and Passalacqua, 2003; Canonica et al., 2014). The protein activity profile of major allergens in body fluids was not reported.

In this section, to estimate the protein activity of JCP-allergen extract after sublingual administration, biological stability of two major allergens of JCP (Cry j 1 and Cry j 2) was analysed by the treatment in human saliva and artificial gastric juice *in vitro* with consideration of SLIT-swallow administration route.

### 3.2 Materials and Methods

The extract was prepared from JCP with identification of including of major allergens. Saliva was pooled from three volunteers in Torii Pharmaceutical Co. Ltd. Artificial gastric juice was prepared by 0.03 mol/L NaCl, 0.084 mol/L HCl and 0.32% (w/v) pepsin with adjustment to pH 1.2.

JCP-allergen extract containing 15 μg/mL Cry j 1 was incubated with saliva or artificial gastric juice in 1 mL tube for 2, 15, 30, 1h, 2h and 4h at 37°C (n=3). The samples at each point were evaluated Cry j 1 and Cry j 2 contents by Enzyme-Linked Immunosorbent Assay (ELISA), total allergic potency by binding to specific IgE in sera of JC pollinosis patients and molecular based-protein profile by SDS-PAGE (data not shown).

Cry j 1 and Cry j 2 contents were measured by sandwich ELISA using anti-Cry j 1 antibody as stabilized antibody and biotin-anti-Cry j 1 antibody as

detection antibody. After peroxidase enzyme reaction, Absorbance 490 nm was detected and concentrations of Cry j 1 or Cry j 2 were calculated.

Total allergenic potency was detected by inhibition ELISA system. Pooled sera from JCP-allergic patient volunteers (Torii Pharmaceutical Co. Ltd.) were used. Coated JCP-allergen extract was incubated with the pooled sera pre-treated with each test samples. Anti-human IgE antibody labelled with β-D-galactosidase reacts to JCP-allergen extract-binding IgE in the well of the microplate. Fluorescence was measured by plate reader. Control value is obtained by pretreatment with buffer. The % inhibition was against control value.

### 3.3 Results and Discussion

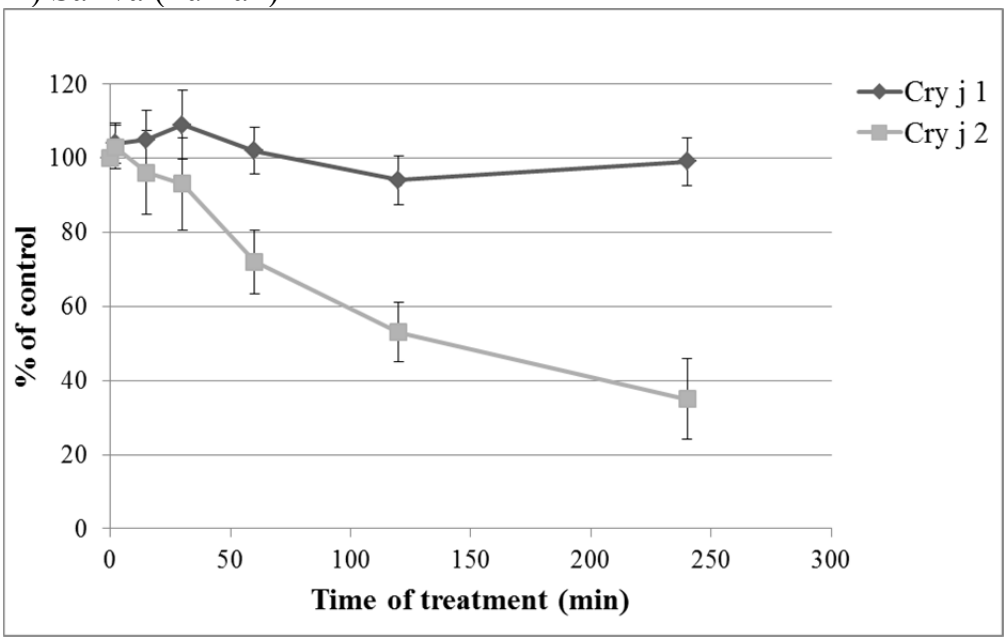
Cry j 1 and Cry j 2 contents by ELISA (antigen-antibody reaction) and total allergic potency (binding to specific antibody in JC pollinosis patients) were measured after treatment in saliva and artificial gastric juice.

In saliva, Cry j 1 and Cry 2 were stable for 2, 15 and 30 min treatment. The content of Cry j 1 was not affected by saliva in 1 h, 2 h and 4 h as maximum treatment time. The content of Cry j 2 was slowly decreased from 30 min to 4 h at less than 40% of initiation. Total allergic potency was not changed by treatment with saliva (Figure 10). On the other hand, in artificial gastric juice, Cry j 1 was disappeared immediately and Cry j 2 was detected approximately 70 % at 0 min followed by decrease to 0% at 15 min and longer time. Total allergic potency was decreased immediately after addition of JCP-allergen extract into artificial gastric juice (Figure 11). The result by SDS-PAGE was consistent with ELISA and total allergic potency (data not shown).

Regarding another allergen, house dust mite, Der f 1 and Der f 2 were investigated on the activity after treatment with body fluids (Du et al., 2011).

For the results mention above, it is suggested that major allergens of JCP-allergen extract could be stable in saliva at sublingual administration in human (2 min). The major allergens are considered to be degraded immediately after the swallowing, and lost allergic potency in gastric juice of a stomach.

A) Saliva (human)




B) Gastric juice (artificial)

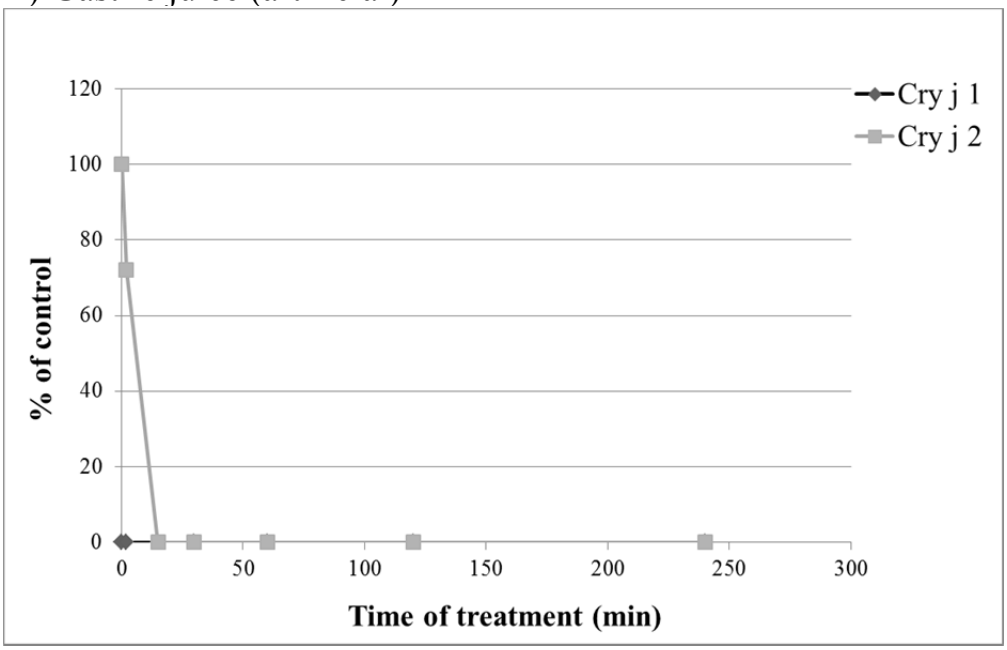
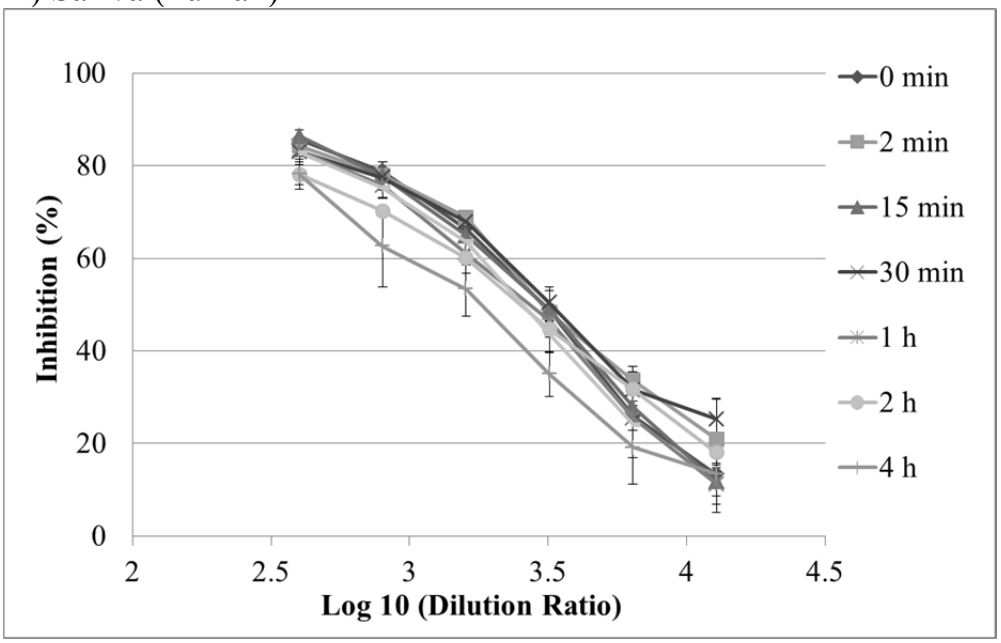



**Figure 10. Major allergen contents after treatment with human saliva and artificial gastric juice.**

JCP-allergen extract was treated with human saliva (A) and artificial gastric juice (B) for 0, 2, 15, 30, 1 h, 2 h and 4 h at 37°C (n=3). Each sample was evaluated Cry j 1 and Cry j 2 concentrations by Cry j 1 specific sandwich ELISA and Cry j 2 specific sandwich ELISA, respectively. The % of control was calculated as the % ratio of Cry j 1 or Cry j 2 concentration compared to control (before treatment).Values are expressed as mean ± SD of tree experiments.

A) Saliva (human)




B) Gastric juice (artificial)

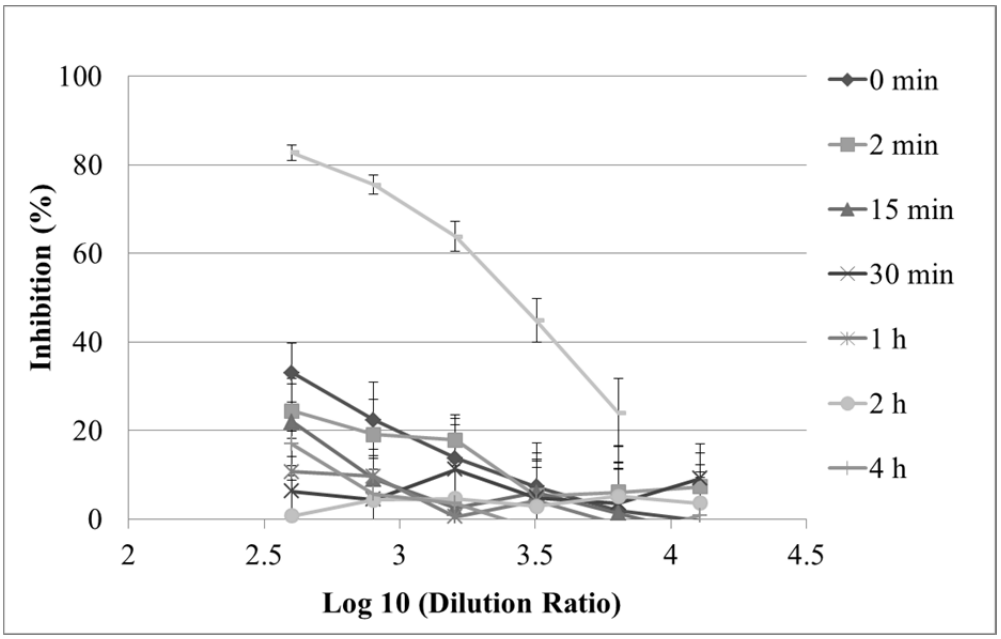



**Figure 11. Total allergic potency after treatment with human saliva and artificial gastric juice.**

JCP-allergen extract was treated with human saliva (A) and artificial gastric juice (B) for 0, 2, 15, 30, 1 h, 2 h and 4 h at 37°C (n=3). Each sample was evaluated total allergic potency by inhibition ELISA. The sera of JCP-allergic patients were pre-treated with the each sample and JCP-allergen extract specific IgE binding was detected as fluorescence. The inhibition % was calculated as the % ratio of fluorescence intensity compared to control (sera pre-treated with buffer).Values are expressed as mean ± SD of tree experiments.

### 3.4 Short Summary

It is suggested that major allergens of JCP-allergen extract could be stable in saliva at sublingual administration (2 min). The major allergens are considered to be degraded immediately after the swallowing, and lost allergic potency in gastric juice of a stomach.

# 4 Immunological Response in Rat induced by Repeated Administration

## 4.1 Introduction

Allergen extract have two sides of immunological properties; antigen profile and allergy profile. The antigen profile is characterized with the potency to change the induction of immunoglobulin. As mentioned in introduction of Chapter II, the changes on immunoglobulin-based immunological reaction (IgG subtype, IgE, and IgA in SLIT) and cell-based immune response induced by repeated administration of allergen extract are key events in mechanism of AIT. Allergen exposure modifies serum levels of allergen-specific IgE and IgG in AIT, shown in Figure 4 in introduction.

To evaluate antigen profile of JCP-allergen extract, I established ELISA test method for detection of specific IgG to Cry j 1 in sera (Figure 12). In this section, specific IgG to Cry j 1 was measured by the established ELISA method following 26 weeks repeated administration. Two different routes as oral administration and subcutaneous administration were compared.

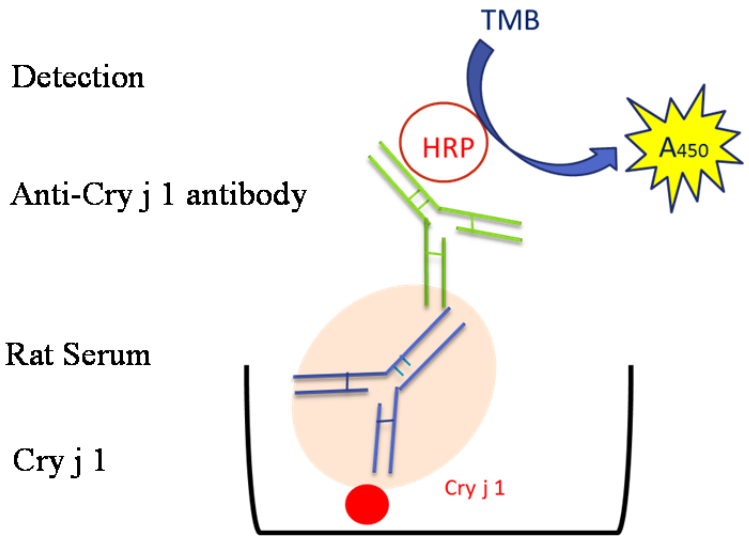



**Figure 12. Detection of Cry j 1 specific IgE by ELISA**

Allergen-specific IgG in rat sera was detected by ELISA system. Cry j 1 specific IgG binds to immobilized Cry j 1 followed by being detected with HRP-conjugate 2nd antibody. Enzyme reaction is used for the final detection.

The serum samples were obtained from 26 weeks repeated toxicological studies by oral and subcutaneous administration studies under GLP compliance, and measurement was conducted separately under non-GLP compliance.

## 4.2 Materials and Methods

The sera samples of rats (n=12) in blood chemistry of 26 weeks repeated toxicological studies were used for analyze of immune response to JCP-allergen extract. The measurement of Cry j 1-specific IgG was conducted in separate study of 26 weeks repeated toxicological studies by oral administration (refer to Chapter III; 200, 2,000, 10,000 JAU/kg) and subcutaneous injection (2,000, 10,000 JAU/kg). These sera were obtained at one day after last dosing at 26 weeks.

Cry j 1-specific IgG in sera were detected by ELISA as below. Microtitre plates (Thermo Fisher Scientific, Inc., Waltham, MA) were coated with 10 μg/mL of Cry j 1 (Torii Pharmaceutical Co. Ltd.) in 100 μL of PBS buffer overnight at 4°C. The plates were washed three times with Tris buffered saline containing 0.05% Tween 20 (T-TBS) and incubated with blocking buffer for 2 h at 25°C. The blocking buffer is PBS containing 5 mg/mL BSA, 1.0 w/v% Block Ace and 0.05 vol% Tween 20. After washing three times, rat sera were added to the wells and incubated for 2 h at 25°C. The anti-Cry j 1 IgG (Asahi Food & Healthcare, Ltd., Tokyo, Japan) was used as positive control. Negative control was sera of normal rats used in 8-fold dilution. After washing three times, HRT-ProteinG was added to the wells for 1 h at 25°C. After washing three times, TMB solution was added and enzyme reaction was stopped with 1 mol/L $H_2SO_4$ after 15 min incubation. The absorbance at 450 nm (A450) was measured by micro plate reader GENios (TECAN Austria GmbH, Grödig, Austria). `Positive` was defined as more than 2-fold A450 of negative control in this study in preliminary study based on statistical analysis (Shankar et al., 2008).

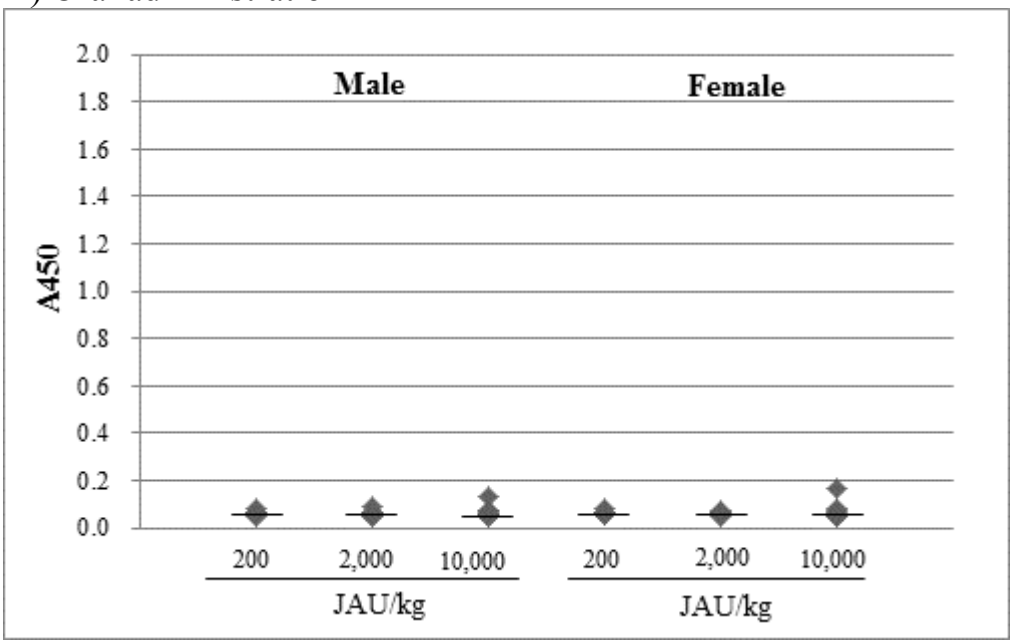
### 4.3 Results and Discussion

In the rat sera with repeated oral administration, no apparent increase was shown compared to negative control (normal sera; A450 = 0.064) and no dose-dependent change was observed in 200, 2,000 and 10,000 JAU/kg of male and female as shown in Figure 13 A (median; 0.058, 0.057, 0.056 respectively in male and 0.058, 0.055, 0.056 respectively in female). Only one male and female in 10,000 JAU/mL showed slight increase and defined as positive.

In the rat sera with repeated subcutaneous injection, all the male and female sample showed higher values than negative control (normal sera; A450 = 0.064). The median values were higher in 10,000 JAU/mL (0.220 in male, 0.207 in female) than 2,000 JAU/mL (0.165 in male, 0.134 in female) as shown in Figure 13 B. The 10 male and 7 female in at 2,000 JAU/kg, 9 male and 9 female at 10,000 JAU/kg were showed positive on Cry j 1-specific IgG production after 26 weeks repeated dosing of JCP-allergen extract.

On the other hand, Cry j 1 concentration in sera was measured in 26 weeks repeated treatment at 0, 13 and 26 weeks at 1, 2, 4, 8, 24 hr after dosing for each time points (data not shown). In oral administration, there was no detection of Cry j 1 after 0, 13 and 26 weeks administration. In subcutaneous injection, Cry j 1 was not detected in 0, 13 weeks and detected in only one female after 26 weeks administration at 1 h (1.52 ng/mL) and 2 h (1.01 ng/mL) time points after administration.

A) Oral administration




B) Subcutaneous administration

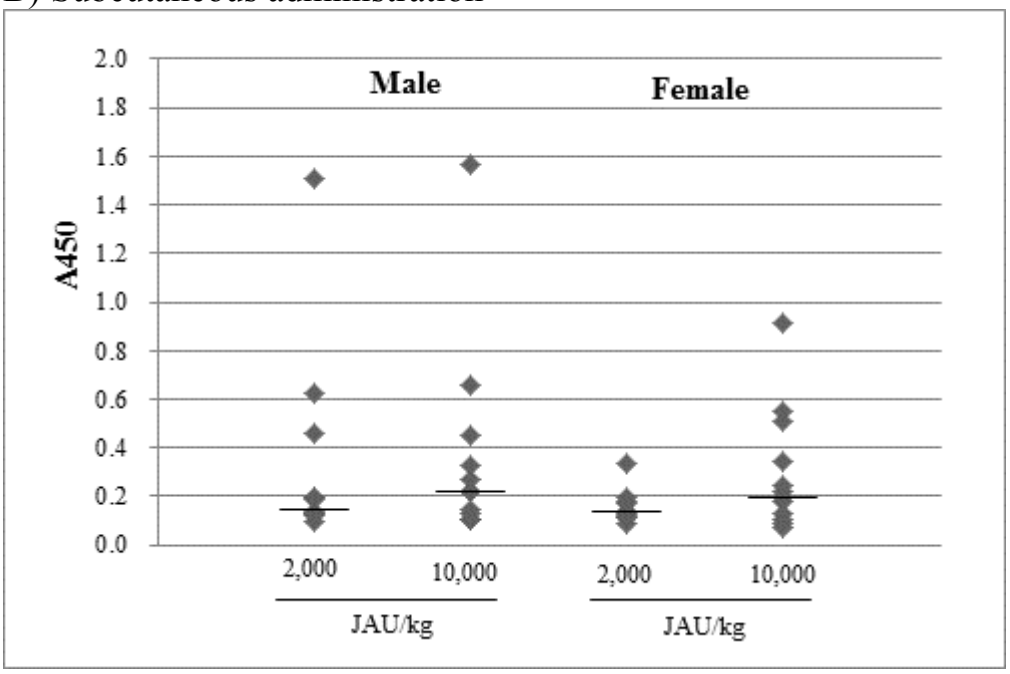



**Figure 13. Cry j 1-specific IgG in rat sera following 26 weeks repeated administration of JCP-allergen extract.**

Rats were administered 200, 2,000 or 10,000 JAU/kg of JCP-allergen extract daily for 26 weeks orally (A) or 2,000 or 10,000 JAU/kg daily for 26 weeks subcutaneously (B). Samples were collected at one day after last dosing at 26 weeks. The dots are individual values of 12 animals per group. The bars indicate median values in each group.

## 4.4 Short Summary

There is a difference on the potency to induce specific IgG between oral and subcutaneous treatment of JCP-allergen extract in rats. The 26 weeks repeated subcutaneous injection of JCP-allergen extract increased Cry j 1 specific IgG in rat sera, although except for one female, Cry j 1 in sera was not detected when during 26 weeks. Oral administration showed no increase in Cry j 1 specific IgG with consistent to no Cry j 1 detection in sera during 26 weeks.

## 5 Summary

Two types of profile of antigen and allergen were investigated with JCP-allergen extract.

The immune reactions induced by JCP-allergen extract were confirmed by PCA reaction in sensitized-model rats. It is suggested that major allergens of JCP-allergen extract are stable in saliva at sublingual administration (2 min), and degraded and lost allergic potency immediately after swallowing in gastric juice of a stomach.

There is a difference on the potency to induce specific IgG between oral and subcutaneous treatment in rats. Subcutaneous injection relatively increased Cry j 1 specific IgG in sera of rats treated 26 weeks repeated dosing, although Cry j 1 in sera was not detected in the study. Oral administration showed no increase in Cry j 1 specific IgG with no Cry j 1 in sera during 26 weeks.

As mentioned above, allergen extract have antigen profile and allergen profile, and it is a key points on use of allergen extract for specific immunotherapy.

# Chapter III. SAFETY EVALUATION ON SYSTEMIC TOXICITY OF REPEATED DOSED RATS WITH ALLERGEN EXTRACT OF JAPANESE CEDAR POLLEN

## 1 Introduction

In the process of the research and development of new drug which is treated long period with repeated administration in human, systemic toxicity of repeated dosed animals is needed to be evaluated in nonclinical study before initiation of clinical trial in long period. The purpose of repeated toxicity study using animals is to examine the potential of the drug to induce toxic changes when repeated administration in long period, providing the dose which induce toxic changes, type and grade of toxicity, No-observed-adverse-effect level (NOAEL) which is the maximal dose indicating no toxicity and No-observed-effect level (NOEL) which is maximal dose indicating no effect compared to normal animals.

For safety evaluation on systemic toxicity of repeated dosing of standardized JCP-allergen extract, 26 weeks repeated study using rodent by oral administration was conducted. Oral administration was selected with considering the route in clinical use, SLIT-swallow. The rats were used for this study because basic information could be provided from single dosed toxicity

and 2 week repeated toxicity (refer to section 3 of Chapter IV) in rats already conducted. The doses in this study were set based on the data in 2 weeks repeated toxicological study in rats.

In Chapter III, systemic toxicology of repeated dosing of JCP-allergen extract during 26 weeks is discussed by the effect of clinical observation, body weight, food consumption, ophthalmoscopy, urinalysis, hematology, blood chemistry, and necropsy, organ weights and histopathology on the last day of 26 weeks dosing period. Dosing period was determined to be 26 weeks according to the guideline on repeated toxicity required for the application to authority. AIT is generally considered to be continued more than 3 years (Bousquet et al., 1998; Canonica et al., 2014), but the ratio of highest dose this study compared to clinical use is sufficient high to evaluate potency of JCP-allergen extract to induce systemic toxicity during repeated dose, it equals to 300-fold (10,000 JAU/kg/day in rat versus 33 JAU/kg/day in human).

This study was conducted according to the Guidelines for Repeated-dose Toxicity Studies in compliance with GLP Regulations specified by the Japanese Ordinance. All procedures employed in the experiments were approved by the Animal Ethics Committee of Mitsubishi Chemical Medience Corporation, Kumamoto, Japan, in accordance with laboratory animal welfare guidelines.

## 2 Materials and Methods

### 2.1 Animals and husbandry

Specific pathogen-free (SPF) Crl:CD (Sprague-Dawley) rats were supplied by Charles River Laboratories (Shiga, Japan) at 4 weeks of age. On arrival, body weights of 92 male and female rats were measured and were within the range of 70.1–90.8 g and 67.5–82.8 g, respectively. All animals were acclimatized to the testing environment for 12–14 days. Dosing of the test articles was initiated at 6 weeks of age. Animals were housed in stainless-steel cages under controlled environmental conditions with temperatures between 22.1–24.5°C, relative humidity of 21.1–77.3%, air ventilation of 10–20 times/h, illumination 12-h per day (light on at 7:00 a.m. and off at 7:00 p.m.), and feed (certified diet CRF-1, Oriental Yeast Co., Ltd.) and water were available *ad libitum.*

### 2.2 Standardized allergen extract of Japanese cedar pollen

Standardized JCP-allergen extract is glycerinated JCP-allergen extract obtained by managed process and controlled by the content of Cry j 1, a major allergen protein of Japanese cedar pollen (Yasueda et al., 1983), and its allergen potency is expressed as Japanese allergen unit (JAU) defined based on skin reaction (Yasueda et al., 1996). Standardized JCP-allergen extract original solution 10,000 JAU/mL (10,000 JAU/mL JCP) is defined to contain 7.3-21 μg/mL of Cry j 1(Yasueda et al., 1996). Test liquids of 200 and 2,000 JAU/mL were formulated by dilution of 10,000 JAU/mL JCP to provide the indicated potency, respectively (Torii Pharmaceutical Co. Ltd., Tokyo, Japan).

In a repeated 26-week oral toxicity study, 200, 2,000 and 10,000 JAU/mL of standardized JCP-allergen extract were used for oral administration. In

addition, 50% glycerin-containing sodium chloride was used for the vehicle control group.

### 2.3 Treatment of test article

Dose levels were selected based on the repeated 2-week oral toxicity study (non-GLP; refer to section 3 of Chapter IV) conducted with the test article. In this study, rats (12/sex-group) received daily oral doses of 200, 2,000 and 10,000 JAU/mL of standardized JCP-allergen extract by gastric gavage at a constant dosing volume of 1 mL/kg for 26 weeks, resulting in 0 (vehicle control group), 200, 2,000, and 10,000 JAU/kg/day (Table 3).

Oral administration was selected for administration, as this is the route used in the clinic (droplets is placed under the tongue, maintained for 2 minutes and then swallowed). In this study, 200 JAU/mL, 2,000 JAU/mL and standardized JCP-allergen extract original solution 10,000 JAU/mL (Torii Pharmaceutical Co. Ltd., Tokyo, Japan) were used. The vehicle consisted of JCP-allergen extract with 50% glycerin containing sodium chloride. The day of administration was defined as ‘day 1’. The week of administration was defined as ‘week 1’.

**Table 3. Experimental design of safety evaluation on systemic toxicity of repeated dosed rat with JCP-allergen extract.**

| Groups | | Treatment | Dosed volume (mL/kg) | Systemic exposure (JAU/kg) | Number of animals | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Main study | | Recovery period [b] | |
| | | | | | M | F | M | F |
| Control | | Saline | 1 | 0 | 12 | 12 | 6 | 6 |
| Vehicle control | | Vehicle [a] | 1 | 0 | 12 | 12 | 6 | 6 |
| JCP | 200 JAU solution | JCP | 1 | 200 | 12 | 12 | 0 | 0 |
| | 2,000 JAU solution | JCP | 1 | 2,000 | 12 | 12 | 0 | 0 |
| | 10,000 JAU solution | JCP | 1 | 10,000 | 12 | 12 | 6 | 6 |

M: male, F: female
JCP: allergen extract of Japanese cedar pollen
[a] 50% Glycerin, 5% Sodium chloride
[b] Four weeks without dosing after 26 weeks dosing period.

## 2.4 Clinical observations, body weights and food consumption

All animals were observed twice daily (before dosing and after dosing) for clinical signs during the dosing period and once daily during the recovery period. Body weights and food consumption were measured immediately before the initiation of dosing on day 1, once a week during the dosing period and the recovery period. A feeding vessel containing food was weighed and set in the cage. The remaining diet was weighed at 24 h to calculate food consumption.

## 2.5 Ophthalmoscopy

Ophthalmoscopy was conducted on all animals from each group before the initiation of dosing: n=12 animals in week 13 and 26 before dosing per

group, and all animals during the recovery period. External eye observations were performed and followed by examination for light reflex using direct ophthalmoscope (Heine Optotechnik, Herrsching, Germany). After observation of the cornea, iris, conjunctiva, lens, and vitreous body using a slit-lamp (SL-15, Kowa Company, Nagoya, Japan), the ocular fundus was examined using binocular indirect ophthalmoscope (Heine Optotechnik)

## 2.6 Urinalysis

Urinalysis was performed for 12 animals per group at weeks 13 and 26 of the dosing period, and for all animals in the recovery period. Fresh urine was collected in the morning before dosing and the following parameters were examined using dipstick analysis: pH, protein, glucose, ketone bodies, bilirubin, occult blood and urobilinogen. Accumulated urine was collected overnight for 24 h and the following parameters were examined: volume, color, osmotic pressure (Osmomat 030-D-RS, Genotec, Berlin, Germany), sodium, potassium and chloride ion concentration (EA07, A&T Corporation, Yokohama, Japan). Urine sediments were microscopically examined for epithelial cells, erythrocytes, leukocytes, casts and crystals.

## 2.7 Hematology and blood chemistry

Hematology was conducted using blood and plasma obtained at necropsy. The animals were fasted for 16–23 h before blood sampling. Ten hematological parameters were measured: numbers of leukocytes and erythrocytes, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, reticulocyte counts, platelets and differential leukocyte counts, using a hematology system (ADVIA

120, Siemens Healthcare Diagnostics, Deerfield, IL). Two hematological parameters—prothrombin time and activated partial thromboplastin time—were determined by an autocoagulometer (Sysmex CA-5000, Sysmex Corporation, Kobe, Japan).

Blood chemistry was conducted using sera obtained at necropsy. Estimations of various biochemical parameters: total protein, total bilirubin, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total cholesterol, triglycerides, phospholipids, glucose, blood urea nitrogen, creatinine, inorganic phosphorus and calcium (Ca), were performed using an auto-analyzer (7170, Hitachi Ltd., Tokyo, Japan). Serum protein fractions (total protein × ratio), albumin and albumin/globulin ratio (A/G ratio) were estimated by an electrophoresis system (AES320, Beckman Coulter, Brea, CA). Sodium (Na), potassium (K) and chloride (Cl) were estimated by an electrolyte analyzer (EA07, A&T).

## 2.8 Necropsy, organ weights and histopathology

Following euthanasia by exsanguination from the abdominal inferior vena cava under pentobarbital anesthesia, all animals were examined in detail for gross lesions. Submandibular glands (with sublingual glands), liver, lungs (with bronchi), thymus, spleen, heart, kidneys, prostate gland, seminal vesicle, testes, epididymis, ovaries, uterus, pituitary, thyroids (with parathyroids), adrenals and brain were weighed after macroscopic examination, and the relative weight of each organ to the final body weight was calculated. For histopathology, after processing sections and staining with hematoxylin and eosin (HE) for light microscopy, the tongue, esophagus, stomach, duodenum, jejunum, ileum (with Peyer's patch), cecum, colon, rectum, parotid gland, pancreas, trachea, lower jaw lymph nodes, mesentery lymph node, aorta (chest), bladder, vagina, mammary

gland, spinal cord (cervical region), optic nerves, sciatic nerve, eyeballs, Harderian glands, biceps femoris muscle, sternum (with bone marrow), femoral fracture (with bone marrow), skin (lower abdomen) and the organs and tissues above that were examined macroscopically were analyzed histopathologically. Organ weights were expressed as relative weights versus body weights (100 g) measured at necropsy day.

## 2.9 Statistical analysis

Statistical analysis was performed for body weight, food consumption, urinalysis hematology, blood chemistry and organ weights using the MiTOX-PPL system (Mitsui Zosen Systems Research Inc., Chiba, Japan). For multiple comparisons, mean values and standard deviations were calculated for vehicle control group and treatment groups. The homogeneity of variances was evaluated using Bartlett's test (significance level: 5%). In the case of homogeneous data, Dunnett's multiple-comparison test was performed to compare with the vehicle control group. In the case of heterogeneous data, Steel's multiple-comparison test was performed to compare with the vehicle control group. In cases of multiple comparisons, the two-sided test was used ($P < 0.05$, $P < 0.01$). For comparisons between two groups, mean values and standard deviations (SD) were calculated for control and vehicle control groups. The homogeneity of variances was evaluated using the F-test (significance level: 5%). For homogeneous data, the Student's t-test was used to compare with the control group. For heterogeneous data, Welch's test was performed to compare with the control group. For comparison between the two groups, a two-sided test was used ($P < 0.05$, $P < 0.01$).

## 3 Results and Discussion

### 3.1 Clinical observations, body weights and food consumption

No test article related mortality was observed in any JCP-allergen extract treatment group and there were no treatment related changes in clinical observations, body weights (Figure 14) and food consumption (Figure 15) during the 26-week dosing period and 4-week recovery period. Non-treatment related changes, callus in hind limb, were observed in one male each from the vehicle control group and 2,000 JAU/kg group.

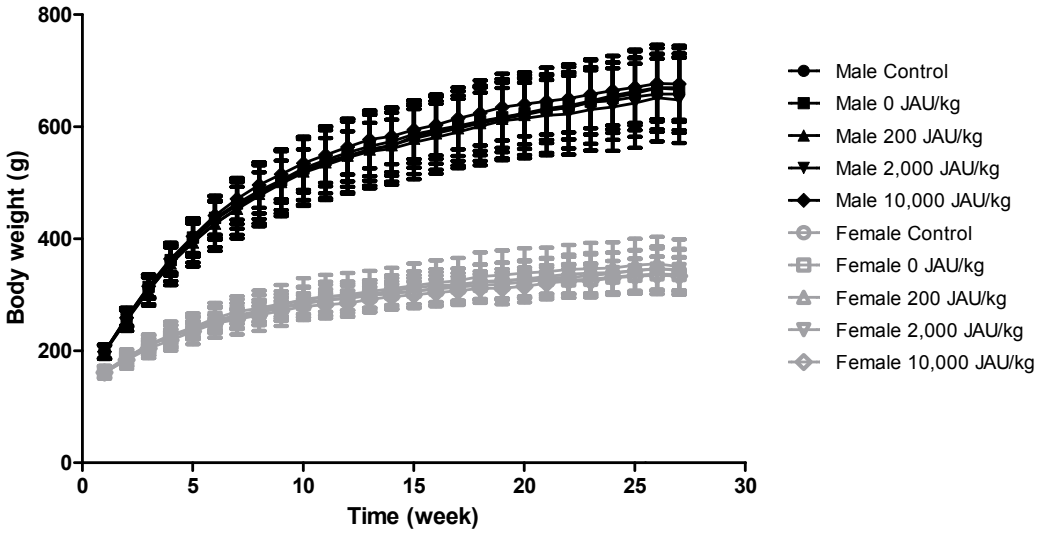



**Figure 14. Body weights of rats following oral administration of JCP-allergen extract.**

Rats were administered 200, 2,000 or 10,000 JAU/kg of JCP-allergen extract or vehicle (0 JAU/kg) daily for 26 weeks. Values are expressed as mean ± SD of 18 animals for all groups except for female control group where n=17 and 200 JAU/kg and 2,000 JAU/kg groups where n=12.

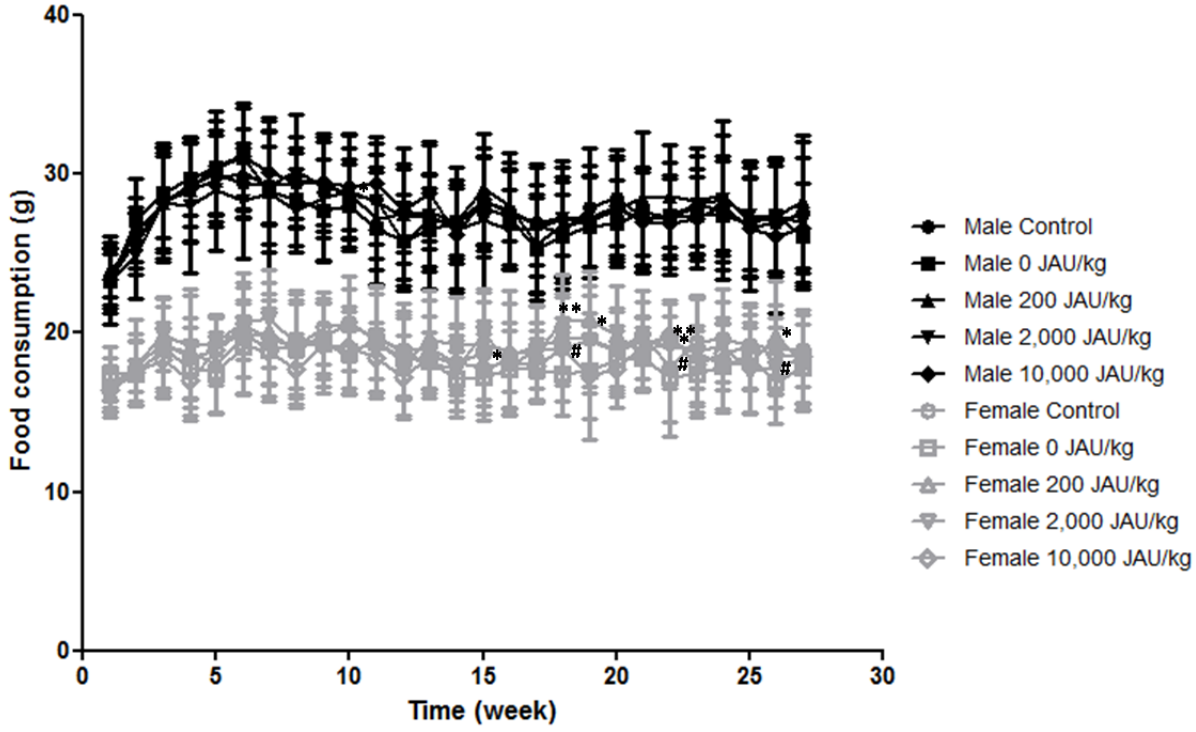



**Figure 15. Food consumption of rats following oral administration of JCP-allergen extract.**

Rats were orally administered 200, 2,000 and 10,000 JAU/kg of JCP-allergen extract or vehicle (0 JAU/kg) daily for 26 weeks. Values are expressed as mean ± SD of 18 animals in all groups except female controls where n=17 and 200 JAU/kg and 2,000 JAU/kg groups where n=12.
#: $p<0.05$ (significantly different from control).
*: $p<0.05$, **: $p<0.01$ (significantly different from 0 JAU/kg).

## 3.2 Ophthalmoscopy and urinalysis

Ophthalmologic examinations did not reveal treatment-related ocular lesions in any of the animals tested (data not shown).

Urinalysis and microscopic examination of the sediment in the vehicle control group indicated increased sodium ions and chloride ions compared with the control group at 13 and 26 weeks, but the findings were not observed in the recovery period. No changes were observed in the JCP-allergen extract treatment groups compared with the vehicle control group at 13 weeks, 26

weeks and the recovery period (data not shown). It was considered that urinalysis indicated that sodium chloride in the vehicle of JCP-allergen extract increased sodium and chloride ion levels compared with the control group. This suggested that increases of sodium and chloride ions by urinalysis have no toxicological significance.

### 3.3 Hematology and blood chemistry

Results for hematology are shown in Table 4. Treatment-related changes were not observed in the JCP-allergen extract treatment group compared with the vehicle control group. The mean platelet counts was significantly increased in 10,000 JAU/kg group male rats but was not considered treatment related because the individual counts of rats (n=12) were within the range measured in the control group. The mean lymphocyte ratio was significantly increased in 10,000 JAU/kg group male rats and the mean of monocyte ratio was significantly decreased in 200 JAU/kg, 2,000 JAU/kg and 10,000 JAU/kg group male rats. These were not considered treatment related changes because no changes were seen in counts of leucocytes and monocytes. Increased numbers of erythrocytes observed in 200 JAU/kg male rats was not dose-dependent.

Results for blood chemistry are shown in Table 5. Treatment-related changes were not observed in JCP treatment groups compared with the vehicle control group. The following changes were not dose-dependent: decreased Cl in 200 JAU/kg females, increased γ-globulin ratio in 2,000 JAU/kg males and decreased A/G ratio, albumin ratio and total bilirubin in 2,000 JAU/kg females. At the recovery period, no treatment related changes in hematology and blood chemistry were observed in the vehicle control group compared with the control group and between the JCP treatment group and vehicle control group (data not shown).

Regarding toxicokinetics, the amounts of Cry j 1, the major JCP allergen, were measured in the serum from rats treated with 10,000 JAU/mL at week 1, week 13 and week 26 before administration and 1, 2, 4, 8 and 24 h after administration. Cry j 1 protein was not detected in the sera at all the time points tested (data not shown), indicating proteins in the JCP-allergen extract administered orally were degraded immediately in the stomach.

## Table 4. Hematological findings of rats following 26 weeks oral administration of JCP-allergen extract.

| Dosage (JAU/kg) | Male | | | | | Female | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Control | 0 | 200 | 2,000 | 10,000 | Control | 0 | 200 | 2,000 | 10,000 |
| Leukocytes ($10^3$/μL) | 9.49±3.43 | 8.71±2.51 | 9.36±2.05 | 9.09±1.32 | 8.09±1.31 | 5.57±1.22 | 4.64#±0.88 | 4.56±0.80 | 4.82±1.25 | 4.95±1.04 |
| Erythrocytes ($10^4$/μL) | 840±47 | 846±24 | 876*±21 | 849±38 | 849±54 | 757±22 | 757±32 | 762±24 | 745±30 | 759±43 |
| Hemoglobin (g/dL) | 14.8±0.8 | 14.7±0.5 | 15.0±0.5 | 14.8±0.5 | 14.7±0.8 | 14.3±0.4 | 14.4±0.8 | 14.5±0.6 | 14.3±0.3 | 14.4±0.6 |
| Hematocrit (%) | 42.4 ±2.2 | 41.9 ±1.3 | 42.9±1.0 | 42.8±1.6 | 42.0±2.0 | 40.4±1.1 | 40.8±2.0 | 41.0±1.3 | 40.2±0.9 | 40.6±1.8 |
| Mean corpuscular volume (fL) | 50.5±1.5 | 49.5±1.0 | 49.1±1.1 | 50.4±1.5 | 49.6±2.4 | 53.4±1.6 | 53.9±1.6 | 53.8±2.0 | 54.0±1.9 | 53.5±1.7 |
| Mean corpuscular hemoglobin:MCH (pg) | 17.6±0.6 | 17.4±0.4 | 17.1±0.6 | 17.5±0.6 | 17.3±0.9 | 18.8±0.7 | 19.1±0.5 | 19.1±1.0 | 19.1±0.7 | 19.0±0.6 |
| MCH concentration (g/dL) | 34.9±0.6 | 35.1±0.3 | 34.8±0.6 | 34.7±0.4 | 34.9±0.5 | 35.3±0.5 | 35.4±0.6 | 35.4±0.8 | 35.5±0.8 | 35.4±0.5 |
| Reticulocytes (%) | 2.1±1.1 | 1.8±0.2 | 1.7±0.3 | 1.8±0.5 | 2.2±1.9 | 1.9±0.4 | 1.9±0.3 | 1.8±0.4 | 1.9±0.2 | 1.9±0.2 |
| Platelets ($10^4$/μL) | 104.9±15.5 | 95.0±6.8 | 97.4±13.1 | 101.8±9.0 | 105.7*±12.0 | 92.9±10.5 | 94.9±8.5 | 88.4±7.2 | 96.7±11.5 | 98.6±7.2 |
| Prothrombin time (sec) | 11.5±1.2 | 11.5±1.5 | 11.6±1.3 | 11.2±0.5 | 11.0±0.4 | 9.5±0.2 | 9.3±0.2 | 9.3±0.2 | 9.4±0.3 | 9.3±0.2 |
| Activated partial thromboplasmin time (sec) | 20.8±1.7 | 20.9±2.2 | 21.2±1.4 | 21.3±1.0 | 21.2±1.4 | 18.4±1.3 | 18.6±1.0 | 18.5±0.8 | 19.2±0.8 | 18.7±1.6 |
| Eosinophils (%) | 1.9±1.0 | 1.9±0.5 | 1.8±0.4 | 1.7±0.6 | 1.9±0.6 | 2.2±1.1 | 2.1±0.7 | 2.0±0.6 | 1.8±0.5 | 1.8±0.8 |
| Neutrophils (%) | 29.3±13.5 | 24.9±8.6 | 20.2±7.5 | 19.4±3.3 | 18.8±4.4 | 20.3±9.4 | 18.2±4.4 | 19±9.4 | 26.4±10.8 | 17.9±7.4 |
| Lymphocytes (%) | 64.7±14.2 | 68.9±8.6 | 74.6±7.7 | 75.5±4.1 | 75.6*±5.2 | 73.2±10.0 | 75.8±4.6 | 75.0±10.2 | 67.3±12.0 | 76.7±8.5 |
| Basophils(%) | 0.5±0.1 | 0.5±0.1 | 0.5±0.1 | 0.5±0.1 | 0.5±0.1 | 0.4±0.2 | 0.5±0.1 | 0.4±0.1 | 0.5±0.1 | 0.4±0.2 |
| Monocytes (%) | 2.7±0.7 | 3.2±0.9 | 2.3*±0.5 | 2.1**±0.6 | 2.3*±0.8 | 3.0±0.7 | 2.5±0.5 | 2.7±0.7 | 3.0±1.2 | 2.6±0.9 |
| Large unstained cells (%) | 1.0±0.6 | 0.8±0.2 | 0.8±0.3 | 0.8±0.5 | 1.0±0.5 | 0.9±0.5 | 0.9±0.6 | 0.9±0.5 | 1.0±0.7 | 0.7±0.4 |

Values are expressed as mean ± SD of 12 animals in each group.
#: $p<0.05$ (significantly different from control).
*: $p<0.05$, **: $p<0.01$ (significantly different from 0 JAU/kg).

**Table 5. Blood chemical findings of rats following 26 weeks oral administration of JCP-allergen extract.**

| Dosage (JAU/kg) | Males | | | | | Females | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Control | 0 | 200 | 2,000 | 10,000 | Control | 0 | 200 | 2,000 | 10,000 |
| Total protein (g/dL) | 5.7±0.2 | 5.8±0.3 | 6.0±0.3 | 5.9±0.2 | 5.9±0.2 | 6.4±0.5 | 6.4±0.4 | 6.5±0.3 | 6.5±0.4 | 6.6±0.3 |
| Albumin (g/dL) | 2.69±0.17 | 2.76±0.14 | 2.83±0.17 | 2.75±0.11 | 2.8 ± 0.16 | 3.65 ±0.40 | 3.68±0.33 | 3.78±0.23 | 3.61±0.33 | 3.79±0.20 |
| A/G ratio | 0.89±0.09 | 0.92±0.07 | 0.91±0.08 | 0.87±0.06 | 0.89 ± 0.08 | 1.33 ±0.13 | 1.38±0.13 | 1.4±0.13 | 1.25*±0.16 | 1.36±0.09 |
| α1-Globulin (%) | 24.2±2.0 | 25.1±1.8 | 24.7±2.3 | 24.6±1.8 | 25.2±2.1 | 17.2±1.1 | 16.5±1.9 | 16.4±1.4 | 17.7±1.1 | 16.9±1.4 |
| α2-Globulin (%) | 5.4±0.8 | 5.1±0.5 | 5.0±0.4 | 5.2±0.5 | 4.9±0.6 | 4.9±0.5 | 4.8±0.6 | 4.9±0.6 | 5.0±0.6 | 4.6±0.6 |
| β-Globulin (%) | 17.5±1.9 | 17.1±0.9 | 17.2±0.8 | 17.5±1.1 | 17.3±0.9 | 14.5±1.8 | 14.2±1.0 | 14.1±1.2 | 15.0±1.9 | 14.3±1.0 |
| γ-Globulin (%) | 5.9±1.6 | 5.0±1.0 | 5.5±0.8 | 6.2*±1.1 | 5.5±1.0 | 6.5±1.0 | 6.6±1.3 | 6.4±0.7 | 7.1±1.1 | 6.7±0.8 |
| Albumin (%) | 47.0±2.6 | 47.7±2.0 | 47.6±2.2 | 46.6±1.9 | 47.1±2.1 | 56.9±2.5 | 57.9±2.3 | 58.2±2.2 | 55.2*±3.1 | 57.6±1.6 |
| Total bilirubin (mg/dL) | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 | 0.0*±0.0 | 0.1±0.1 |
| Aspartate aminotransferase (IU/L) | 90±31 | 83±10 | 79±17 | 81±26 | 78±8 | 103±58 | 103±36 | 148±159 | 146±142 | 106±56 |
| Alanine aminotransferase (IU/L) | 29±16 | 21±4 | 23±6 | 26±19 | 21±4 | 36±21 | 35±18 | 53±57 | 56±64 | 40±24 |
| Alkaline phosphatase (IU/L) | 138±33 | 123±13 | 124±24 | 120±24 | 130±33 | 53±12 | 51±12 | 52±11 | 54±21 | 51±12 |
| Total cholesterol (mg/dL) | 85±19 | 78±14 | 87±21 | 76±11 | 89±16 | 94±25 | 86±12 | 98±19 | 100±20 | 91±9 |
| Triglycerides (mg/dL) | 78±39 | 76±31 | 92±33 | 67±25 | 87±47 | 39±18 | 41±21 | 43±15 | 43±28 | 45±27 |
| Phospholipids (mg/dL) | 140±24 | 131±15 | 143±23 | 127±15 | 142±22 | 182±39 | 172±25 | 189±30 | 190±35 | 182±21 |
| Glucose (mg/dL) | 125±13 | 128±12 | 126±9 | 123±8 | 126±23 | 119±6 | 117±13 | 116±6 | 117±6 | 120±9 |
| Blood urea nitrogen (mg/dL) | 16.2±3.7 | 16.5±3.2 | 14.8±2.0 | 15.8±2.1 | 16.9±1.4 | 16.1±2.4 | 17.8±2.9 | 16.3±2.4 | 15.3±1.7 | 17.1±3.4 |
| Creatinine (mg/dL) | 0.4±0.1 | 0.4±0.1 | 0.4±0.0 | 0.4±0.1 | 0.4±0.0 | 0.5±0.1 | 0.5±0.1 | 0.5±0.1 | 0.5±0.0 | 0.5±0.1 |
| Inorganic phosphorus (mg/dL) | 6±0.7 | 5.9±0.7 | 5.7±0.6 | 6.0±0.4 | 5.9±0.6 | 4.5±0.7 | 4.6±0.9 | 4.9±0.8 | 4.8±0.9 | 4.9±1.0 |
| Ca (mg/dL) | 10.2±0.3 | 10.2±0.3 | 10.4±0.3 | 10.2±0.3 | 10.4±0.3 | 10.5±0.3 | 10.4±0.4 | 10.6±0.3 | 10.5±0.2 | 10.5±0.2 |
| Na (mEq/L) | 146±0.8 | 145.9±0.7 | 145.8±0.9 | 146.4±0.9 | 145.9±1.0 | 144.4±0.7 | 144.6±0.9 | 143.6±1.3 | 144.6±1.1 | 144.9±0.9 |
| K (mEq/L) | 4.51±0.18 | 4.53±0.28 | 4.42±0.16 | 4.57±0.17 | 4.67±0.21 | 3.89±0.21 | 4.05±0.21 | 3.95±0.21 | 4.0±0.23 | 3.99±0.32 |
| Cl (mEq/L) | 106.4±1.4 | 106.1±1.5 | 105.9±1.1 | 106.4±1.3 | 106±1.1 | 106.8±1.5 | 107.6±1.5 | 106.2*±1.1 | 107.2±0.8 | 107.4±1.1 |

Values are expressed as mean ± SD of 12 animals in each group.
*: $p<0.05$ (significantly different from 0 JAU/kg)

### 3.4 Necropsy, organ weights and histopathology

No gross abnormalities were detected for rats in any group by necropsy at the treatment period and recovery period (Table 6). Callus in hind limbs were observed in one male each from the vehicle control group and 2,000 JAU/kg group corresponding to the clinical observations. A yellow-brown neoplasm was observed in the abdominal cavity of a male rat in the control group.

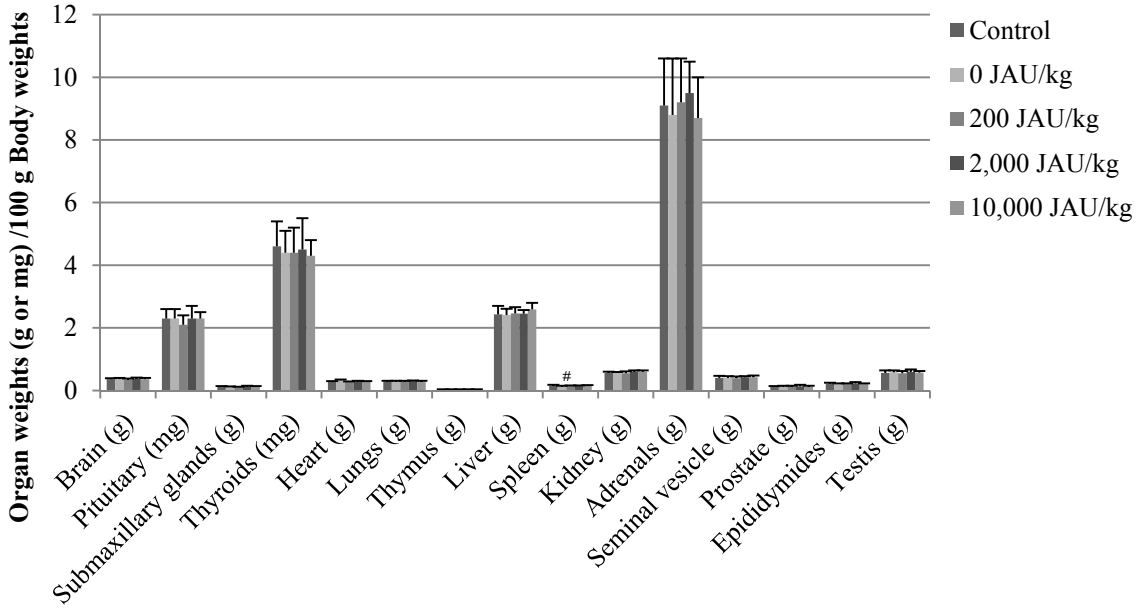
**Table 6. Findings in necropsy of rats following 26 weeks oral administration of JCP-allergen extract.**

| Dosage (JAU/kg) | | Male | | | | | Female | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Control | 0 | 200 | 2,000 | 10,000 | Control | 0 | 200 | 2,000 | 10,000 |
| Others | | | | | | | | | | | |
| Abdominal cavity | Mass, Yellow -brown | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Extremity | Corn, hindlimb | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |

Values are expressed as the number of animals observed with indicated findings in 12 animals of each group.
No appreciable changes in all other organs and tissues.

There were no treatment-related differences in organ weights between the vehicle control and 10,000 JAU/kg groups (Figure 16). A decrease in relative organ weight of the thymus was observed in 200 JAU/kg group females and increased relative organ weight of the kidney (left) was observed in 2,000 JAU/mL males. These were not dose-dependent changes.

A) Male




B) Female

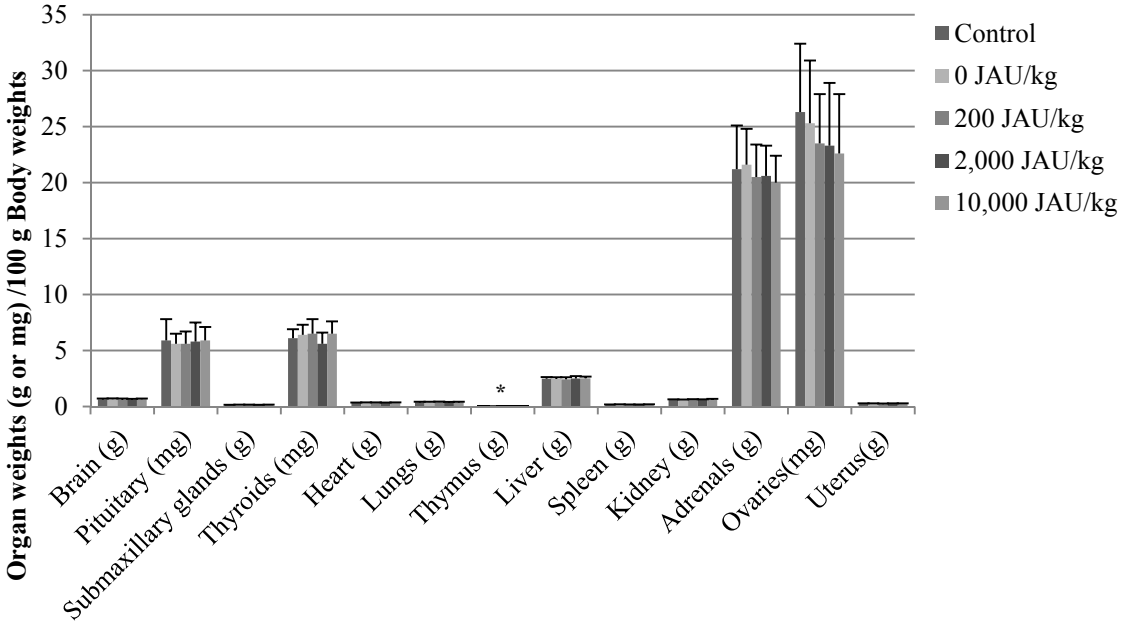



**Figure 16. Organ weights of rats following 26 weeks oral administration of JCP-allergen extract.**

Rats were orally administered 200, 2,000 and 10,000 JAU/kg of JCP-allergen extract or vehicle (0 JAU/kg) daily for 26 weeks. Values are expressed as mean ± SD of 12 animals in each group. #: $p<0.05$ (significantly different from control). *: $p<0.05$ (significantly different from 0 JAU/kg).

Results for histopathology are shown in Table 7. Histopathology revealed no systemic toxicological changes in the organs of vehicle and 10,000 JAU/kg groups, although some changes in organs occurred sporadically. Except for the stomach findings, these changes were considered incidental because they occurred in the control or vehicle control groups, or with a comparable frequency and similar level to the control group.

The stomach is the first organ to come into contact with JCP-allergen extract administered orally. Hyperplasia in the forestomach and increased globule leukocytes in the glandular stomach were observed at comparable levels between the vehicle control group and 10,000 JAU/kg group. The stomach findings disappeared in recovery period suggesting temporary changes. Histopathology at the recovery period indicated no changes (data not shown). Regarding hyperplasia, it might be caused by irritation from glycerin (Staples et al., 1967) in the vehicle solution because it was not observed in the control group administered saline and the frequency and grade of hyperplasia were comparable between vehicle and 10,000 JAU/mL treated group. Of note, it is not appropriate to extrapolate the findings observed in the forestomach, a unique organ in rodents, to potential local changes in the stomach of humans. Regarding globule leukocytes, it is a large intra-epithelial cell, which is thought to be derived from a common precursor of mast cells (Ikeda and Yamashita, 1993), arising during intestinal nematode infections in rodents and ruminants and with granulated lymphocytes (Huntley, 1992). The mild increase of globule leukocytes in the glandular stomach observed in this study might be caused by irritation from glycerin in the vehicle solution. In addition, increased numbers of globule leukocytes are associated with the presence of lesions in the GI tract (Narama et al., 1999) .These findings occurred in the stomach of rats of the repeated 26-week oral toxicity study were toxicologically mild and were not

observed after the 4-week recovery period in the control and JCP-allergen extract treatment groups.

**Table 7. Histopathological findings of rats following 26 weeks oral administration of JCP-allergen extract.**

| Organ/Tissue | Finding | Dose (JAU/kg/day) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Male | | | Female | | |
| | | Control (Saline) | 0 | 10,000 | Control (Saline) | 0 | 10,000 |
| Esophagus | | 0 | 0 | 0 | 0 | 0 | 0 |
| Stomach | Hyperplasia,squamous epithelium,forestomach,limiting ridge | 0 | 3 | 4 | 0 | 4 | 3 |
| | Cyst,glandular stomach | 0 | 0 | 0 | 1 | 1 | 0 |
| | Erosion,glandular stomach | 0 | 1 | 1 | 0 | 1 | 0 |
| | Globule leukocyte,increased,glandular stomach | 0 | 3 | 5 | 0 | 2 | 1 |
| Sublingual gland | Cellular infiltration,lymphocyte | 0 | 0 | 0 | 0 | 0 | 1 |
| Parotid gland | Cellular infiltration,lymphocyte | 3 | 2 | 2 | 1 | 1 | 1 |
| Liver | Hemorrhage,focal | 0 | 0 | 2 | 0 | 0 | 0 |
| | Necrosis,hepatocyte,focal | 0 | 0 | 0 | 1 | 1 | 0 |
| | Cellular infiltration,mononuclear cell | 7 | 5 | 7 | 2 | 2 | 2 |
| | Fibrosis,focal | 1 | 1 | 0 | 0 | 0 | 0 |
| Pancreas | Atrophy,acinus | 3 | 0 | 3 | 0 | 0 | 0 |
| | Focus,acinar cell,basophilic | 1 | 0 | 0 | 0 | 0 | 0 |
| | Deposit,pigment,yellow-brown | 2 | 0 | 2 | 0 | 0 | 0 |
| | Cellular infiltration,eosinophil | 0 | 1 | 1 | 1 | 0 | 0 |
| | Cellular infiltration,lymphocyte | 4 | 5 | 1 | 2 | 5 | 4 |
| | Fibrosis,islet | 2 | 1 | 3 | 0 | 0 | 0 |
| Lung | Metaplasia,osseous | 2 | 0 | 1 | 0 | 0 | 0 |
| | Accumulation,foam cell | 1 | 2 | 3 | 2 | 2 | 1 |
| | Mineralization,artery | 1 | 2 | 2 | 2 | 3 | 4 |
| Spleen | Hematopoiesis,extramedullary | 1 | 0 | 2 | 0 | 0 | 0 |
| Bone marrow (femur) | Hematopoiesis,increased | 1 | 0 | 0 | 0 | 0 | 0 |
| Heart | Cellular infiltration,mononuclear cell | 3 | 3 | 0 | 0 | 1 | 0 |
| Kidney | Tubule,basophilic | 3 | 5 | 3 | - | - | - |
| | Deposit,pigment,tubule,yellow-brown | 1 | 0 | 2 | - | - | - |
| | Cast,proteinaceous | 2 | 3 | 3 | - | - | - |
| | Cellular infiltration,pelvis,inflammatory | 1 | 1 | 0 | 0 | 0 | 1 |
| | Cellular infiltration,lymphocyte | 6 | 5 | 7 | 0 | 1 | 3 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Cyst | | | | 1 | 1 | 0 |
| | Mineralization | 3 | 1 | 2 | 1 | 1 | 0 |
| Urinary bladder | Cellular infiltration,submucosa,lymphocyte | 1 | 0 | 0 | 0 | 0 | 0 |
| Testis | | 0 | 0 | 0 | - | - | - |
| Epididymis | | 0 | 0 | 0 | - | - | - |
| Prostate | Cellular infiltration,lymphocyte | 7 | 2 | 7 | - | - | - |
| Ovary | Cyst | - | - | - | 4 | 3 | 5 |
| Mammary gland | Mineralization | 1 | 0 | 0 | 0 | 0 | 0 |
| | Hyperplasia,lobular | 0 | 0 | 0 | 1 | 0 | 0 |
| Pituitary | Hyperplasia,focal | 1 | 0 | 0 | 0 | 0 | 0 |
| | Cyst | 0 | 1 | 0 | 2 | 0 | 0 |
| Thyroid | Remnant,ultimobranchial body | 1 | 1 | 2 | 1 | 4 | 2 |
| | Hyperplasia,C cell,focal | 0 | 0 | 0 | 1 | 0 | 0 |
| | Cyst | 0 | 0 | 0 | 0 | 1 | 1 |
| Adrenal | Vacuolation,cortical cell,zona fasciculata,focal | 1 | 0 | 0 | 0 | 0 | 0 |
| Cerebellum | Cellular infiltration,chorioid plexus,lymphocyte | 0 | 0 | 0 | 1 | 0 | 0 |
| Eye | Mineralization,cornea | 1 | 1 | 2 | 0 | 0 | 0 |
| Harderian gland | Cellular infiltration,lymphocyte | 2 | 1 | 2 | 4 | 5 | 1 |
| Integument | Crust | 0 | 0 | 1 | 0 | 0 | 0 |

Groups contained 12 rats. The organs and tissues of the 200 JAU/mL and 2,000 JAU/mL groups were not examined. The organs and tissues of 200 JAU/mL and 2,000 JAU/mL group are not examined. .No appreciable changes in all other organs and tissues.

## 4 Summary

A 26-week repeated oral dose toxicity study in rats was conducted with standardized JCP-allergen extract to evaluate its safety for application to SLIT. The route of dosing was selected based on the human therapeutic route of JCP-allergen extract that is SLIT-swallow. Rats were administered JCP-allergen extract at doses of 200, 2,000 and 10,000 JAU/kg-day by body weight. The effect of JCP-allergen extract was compared with a vehicle control group (50% glycerin containing sodium chloride) to evaluate the test substance and the vehicle control group was compared with control group (saline) to evaluate the vehicle.

There were no JCP-allergen extract related toxicologically relevant changes in clinical signs, body weight change, food consumption, ophthalmoscopy, urinalysis, hematology, blood chemistry, necropsy, organ weights and histopathology although slight local changes caused by irritation from the vehicle were observed at the administration site.

Therefore, NOAEL was greater than 10,000 JAU/kg/day for systemic toxicity, equivalent to 300-fold the human dose.

# Chapter IV. LOCAL IRRITATION OF ALLERGEN EXTRACT OF JAPANESE CEDAR POLLEN

## 1 Introduction

In SLIT, the allergen extract is placed under the tongue for 1 or 2 minutes and then swallowed (SLIT-swallow) or spat out (SLIT-spit). Based on clinical results and pharmacokinetic considerations, only SLIT-swallow is currently performed (Canonica and Passalacqua, 2003). Oral irritative reactions are distinctive adverse events in SLIT that include stomatitis and throat irritation induced in the early stages of treatment, in response to the allergen, which activates the immune system. Stomatitis and throat irritation were reported as adverse events in a clinical trial using a sublingual tablet of timothy pollen extract in Europe (Nelson et al., 2011).

In the case of standardized JCP-allergen extract, the allergen droplet is placed under the tongue for 2 minutes and swallowed (SLIT-swallow). Based on such as clinical usage, two selected sites which are oral area and GI tract were examined in local irritation studies. Oral mucosa is first-of-contact site of allergen extract administered sublingual administration because of generally used species for irritation study as well as dogs. Mucosa of GI tract is contact site after swallowing of allergen extract in SLIT. Rats were used for irritation study of GI tract after oral administration because same species was used in 26 weeks repeated toxicological study conducted by oral administration.

In Chapter IV, the potential of standardized JCP-allergen extract to cause oral mucosal irritation was mentioned first in the study of rabbits by repeated sublingual dosing for 7 days of 2,000 JAU/mL, the maximum dose used in the clinic (Mitobe et al., 2012). The optimal volume of administration was determined in a preliminary study to ensure the maximum volume did not leak from the mouth or accidentally be ingested. The administration time to the sublingual area of rabbits was 20 min. It was longer than the 2 min used for human clinical application because the increased grade of lesion is dependent on administration time in oral mucosal irritation studies using rabbits (Ohbayashi et al., 1988).

After that, the potential to cause GI tract irritation was also discussed in the study of rats by repeated oral dosing for 14 days of 10,000 JAU/mL (stocked solution of standardized JCP-allergen extract 2,000 JAU/mL and 200 JAU/mL) (Mitobe et al., 2011). Mucosal irritation of JCP-allergen extract in GI tract was evaluated by necropsy and histopathology conducted on last day of repeated dosing, compared with the vehicle control groups. In addition at this study, systemic toxicity was evaluated in same animals as well as local irritation in GI tract, clinical observation, body weight, food consumption, hematology, blood chemistry, necropsy, organ weight and histopathology. The data obtained from systemic toxicological examination was considered for dose setting in 26 repeated toxicological study by oral administration in the rats.

The study for oral mucosal irritation in the rabbits was conducted in compliance to GLP regulations specified by the Japanese Ordinance. Both studies of oral mucosal irritation in the rabbits and mucosal irritation of GI tract

in the rats were conducted with approval of the Institutional Animal Care and Use Committee of Mitsubishi Chemical Medience Corporation in accordance with laboratory animal welfare guidelines.

## 2 Irritation to Oral Mucosa in Rabbits

### 2.1 Materials and Methods

#### (1) Animals and husbandry

SPF male Kbs: New Zealand White rabbits of 17 weeks of age were supplied by Kitayama Labes Co., Ltd. (Nagano, Japan). Upon arrival, ten male rabbits were weighed (range of 2,829–3,315 g). All animals were acclimatized to the testing environment for 6 days. Dosing of the test article was initiated at 18 weeks of age. The body weight range at initiation of dosing was 3,056–3,485 g. Animals were housed in stainless-steel cages under controlled environmental conditions with temperatures between of 23.1–24.4°C, relative humidity of 47.6–61.6%, air ventilation of 10–20 times/h, illumination 12-hour per day (light on at 7:00 a.m. and off at 7:00 p.m.), and feed (LRC6, Oriental Yeast Co., Ltd.) and water were available *ad libitum*..

#### (2) Standardized allergen extract of Japanese cedar pollen

Standardized JCP-allergen extract is glycerinated JCP-allergen extract obtained by managed process and controlled by the content of Cry j 1, a major allergen protein of Japanese cedar pollen (Yasueda et al. 1983), and its allergen potency is expressed as Japanese allergen unit (JAU) defined based on skin reaction (Yasueda et al., 1996). Standardized JCP-allergen extract original solution 10,000 JAU/mL (10,000 JAU/mL JCP) is defined to contain 7.3-21μg/mL of Cry j 1(Yasueda et al., 1996). Test liquids of 200 and 2,000 JAU/mL were formulated by dilution of 10,000 JAU/mL JCP to provide the indicated potency, respectively (Torii Pharmaceutical Co. Ltd., Tokyo, Japan).

For the oral mucosal irritation study, 2,000 JAU/mL of standardized JCP-allergen extract was administered sublingually, as the maximum dose used in the maintenance phase of SLIT in JC pollinosis.

**(3) Treatment of test article**

Three male rabbits per group received daily sublingual administration for 7 days under sedation by intramuscular injection of medetomidine, with saline (control group), 50% glycerin solution containing sodium chloride (vehicle control group) or JCP-allergen extract (2,000 JAU/mL). The 0.2 mL test solution was administered to the sublingual area for 20 min followed by washing with 30 mL of saline. The 2,000 JAU/mL was used in this study which is maximum dose in the maintenance phase of SLIT in JC pollinosis. The dosing volume of 0.2 mL/head was the maximum technical dose for sublingual administration to rabbits, resulting in 400 JAU/head.

Sublingual administration was selected as it is the intended clinical route.

**(4) Clinical observations and body weights**

All animals were observed twice daily (before dosing and after dosing) for clinical signs during the dosing period (from days 1 to 7) and once at day 8. Body weights were measured immediately before the initiation of dosing on day 1 and on the last day of dosing (day 8).

### (5) Macroscopic observation of administration site

Macroscopic observation of the administration site was performed under sedation before administration from days 1 to 8. The administration site in the oral cavity was estimated for irritative scores according to Ohbayashi's criteria in rabbits (Ohbayashi et al., 1988) shown in Table 8.

**Table 8. Scale for scoring acute oral reactions in the rabbits sublingual mucosa under macroscopic observation.**

| Findings in administration site | Score |
|---|---|
| No reaction | 0 |
| Slight erythema (barely perceptible) | 1 |
| Clear erythema (well defined erythema) | 2 |
| Slight white fur (barely perceptible) | 3 |
| Mild white fur (well defined white fur) | 4 |
| Moderate white fur (thick white fur raised less than one half) | 5 |
| Severe white fur (thick white fur raised more than one-half) | 6 |

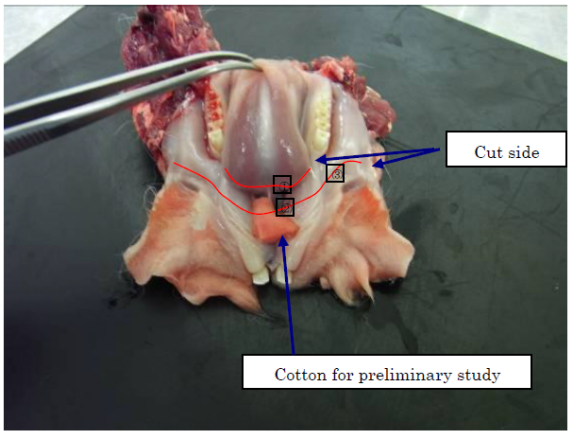
Ohbayashi et al., 1988

### (6) Histopathology of administration site

Histopathology was conducted at the administration site in the oral area of the rabbits. At day 8 after clinical observation, all rabbits were anesthetized with intravenous injection of sodium pentobarbital (30 mg/kg) followed by macroscopic observation of administration site, and euthanasia was performed. Oral mucosal tissues including tongue, mouth floor and buccal area (Figure 17) were removed and fixed in 10 vol% neutral buffered formalin. The mucosa

tissues were embedded in paraffin, sectioned, stained with HE and examined microscopically

A) Photo of oral area of rabbit




B) Corresponding cartoon

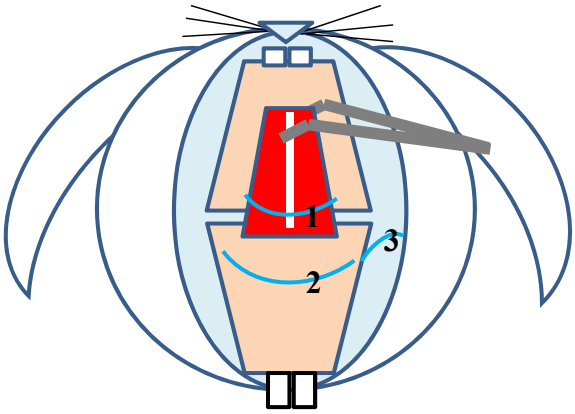



**Figure 17. Administration site including sublingual, mouth floor and buccal area of rabbit.**

The numbers in photo abd cartoon mean 1; sublingual, 2; mouth floor, 3; buccal area. Red lines in photo and blue lines in cartoon show cut side for histopathology. This photo was taken in preliminary study.

## 2.2 Results and Discussion

We confirmed the irritative response caused by positive control observed in the oral mucosa of rabbits administered sublingually for 7 days as preliminary study. Positive control, 35% SLS, showed slight white fur (score 3) in macroscopic observation from day 2 to day 7. Histopathological analysis was conducted on day 8 and H&E straining showed slight erosion and invasion of heterophil in mucosa of mouth floor (Figure 18 A).

Oral mucosal irritation of JCP-allergen extract was evaluated with repeated sublingual dose study in rabbits. There were no treatment-related changes in the clinical observations and body weight of treated rabbits during the treatment of 2,000 JAU/mL of JCP-allergen extract (Table 9).

**Table 9. Macroscopic observations of sublingual/buccal cavity of rabbits following 7 days sublingual administration of JCP-allergen extract.**

| | Body weights (g) | | Scoring of oral mucosa | |
|---|---|---|---|---|
| | **Day 1** | **Day 8** | **Day 1** | **Day 8** |
| Control (Saline) | 3197 ± 185 | 3206 ± 254 | 0 ± 0 | 0 ± 0 |
| Vehicle | 3295 ± 219 | 3244 ± 200 | 0 ± 0 | 0 ± 0 |
| JCP 2,000 JAU solution | 3195 ± 152 | 3132 ± 86 | 0 ± 0 | 0 ± 0 |

Values are expressed as mean ± SD of three animals in each group.
JCP 2,000 JAU solution: Standardized JCP-allergen extract with allergen potency of 2,000 JAU/mL

The macroscopic observation of the administration site revealed no irritation in the sublingual mucosa of animals throughout the dosing period. In addition, histopathology of oral mucosal tissues including tongue, buccal and mouth floor revealed no abnormalities in the treatment groups, although there was mild lymphocytic infiltration in the mucosa of the mouth floor of one animal in the control group (Figure 18B).

A) Positive control; 35%SLS

B) JCP treatment

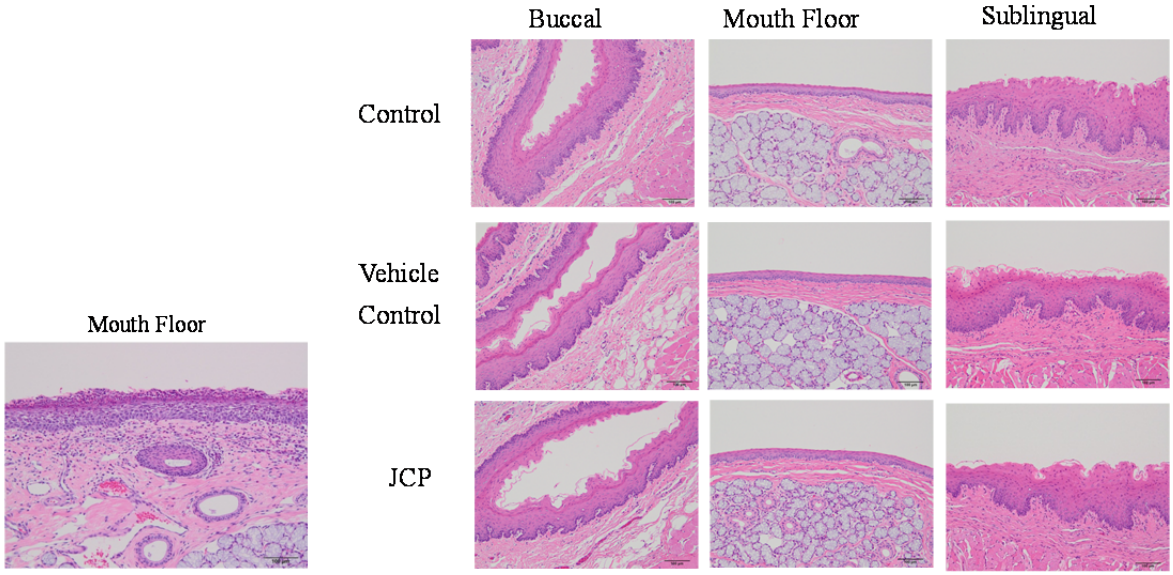



**Figure 18. H&E staining of oral mucosa sections from rabbits following 7 days sublingual administered of JCP-allergen extract.**

Three rabbits/group were administered A) 0.1 mL of 35 w/v% sodium lauryl sulphate (SLS) solution for 10 min sublingually for 7 days and B) 0.2 mL of 2,000 JAU/mL JCP-allergen extract (400 JAU/head), vehicle (0 JAU/head) or saline sublingually for 20 min daily in 7 days. The bars indicate 100 μm.

Irritation study is one of the toxicological studies in the study package for new drug application to authority. The purpose of the study is to evaluate a physical property of the drug its self, so normal animals are used for the investigation. Other burden allergen with induction of allergic asthma and

rhinitis, house dust mite, was evaluated on oral mucosal irritation in the normal dog. No changes were observed up to 60 mg/site/day for 28 days repeated sublingual administration (Ohashi-Doi et al., 2012).

Local irritative response is main adverse event observed in SLIT with allergic patients. In the aspect of character of allergen extract such as local exposure and allergic potency, induction of local reaction in administration area of sensitized animal or human are quite reasonable. In fact, several local irritative reactions such as oral edema, stomatitis, irritation of pharynx and itchiness in mouth were reported in the clinical trial with standardized JCP-allergen extract (Torii Pharmaceutical Co. Ltd., Pharmaceutical interview form; CEDARTOLEN® Sublingual Immunotherapy Droplet of Japanese Cedar Pollen). Further studies are required to examine local irritation in sensitized animals for evaluation of the irritation in allergic patients.

## 2.3 Short Summary

The potential of JCP-allergen extract to cause oral mucosal irritation was evaluated in rabbits by repeated dosing for 7 days of 2,000 JAU/mL, the maximum dose used in the clinic. We confirmed that irritative response of positive control (35% SLS solution) was induced in the oral mucosa of rabbit following sublingually repeated treatment for 7 days. In this study, the volume of administration was 0.2 mL as maximum volume did not leak from the mouth or accidentally be ingested. The administration time to the sublingual area of rabbits was 20 min which is ten-fold longer than the 2 min used for human clinical application.

There were no treatment-related changes by macroscopic observation of the administration site throughout the dosing period and by histopathology of oral mucosal tissues including tongue, buccal and mouth floor. It suggested no local irritation of the sublingual/buccal mucosa occurred in healthy rabbits after 1 week repeated sublingual treatment with JCP-allergen extract drug product with 10-times longer application than for clinical use. Further study is required in JCP-allergen extract -sensitized animals to evaluate the local irritation that appeared during SLIT in patients with JC pollinosis.

## 3 Irritation to Mucosa of Gastrointestinal Tract in Rats

### 3.1 Materials and Methods

#### (1) Animals and husbandry

Specific pathogen-free (SPF) Crl:CD (Sprague-Dawley) rats were supplied by Charles River Laboratories (Shiga, Japan) at 5 weeks of age. On arrival, body weights of 20 male rats were measured and were within the range of 117.3–134.9 g. All animals were acclimatized to the testing environment for 5 days. Dosing of the test articles was initiated at 6 weeks of age. Animals were housed in stainless-steel cages under controlled environmental conditions with temperatures between 23.3–24.5°C, relative humidity of 43.6–62.0%, air ventilation of 10–20 times/h, illumination 12-h per day (light on at 7:00 a.m. and off at 7:00 p.m.), and feed (certified diet CRF-1, Oriental Yeast Co., Ltd.) and water were available ad libitum.

#### (2) Allergen extract of Japanese cedar pollen

Standardized JCP-allergen extract is glycerinated JCP extract obtained by managed process and controlled by the content of Cry j 1, a major allergen protein of Japanese cedar pollen (Yasueda, et al. 1983), and its allergen potency is expressed as Japanese allergen unit (JAU) defined based on skin reaction (Yasueda et al., 1996). Standardized JCP-allergen extract original solution 10,000 JAU/mL (10,000 JAU/mL JCP-allergen extract) is defined to contain 7.3-21μg /mL of Cry j 1(Yasueda et al. 1996).

For the irritation study to mucosa of GI tract in rat, 10,000 JAU/mL JCP-allergen extract was administered orally in each dose levels.

### (3) Treatment of test article

Three male rats per group received daily oral administration of 10,000 JAU/mL of standardized JCP-allergen extract by gastric gavage at a constant dosing volume of 0.1, 0.5 and 2.5 mL/kg for 14 days, resulting in 200, 2,000, and 1,000, 5,000 and 25,000 JAU/kg/day (Table 10). Animals of vehicle control group received daily oral administration of 50% glycerin solution containing sodium chloride in same way as treated group in 0.1, 0.5 and 2.5 mL/kg respectively, for each controls. Oral administration route was selected as it is the intended clinical route. The day of administration was defined as 'day 1'.

**Table 10. Experimental design of irritation study to mucosa of gastrointestinal tract in rats**

| Groups | | Treatment | Dosed volume (mL/kg) | Exposure (JAU/kg) | | Number of animals (Male) |
|---|---|---|---|---|---|---|
| | | | | Systemic ( /kg) | Local[c] ( /site) | |
| Vehicle control | -1 | Vehicle[a] | 0.1 | 0 | 0 | 3 |
| | -2 | Vehicle[a] | 0.5 | 0 | 0 | 3 |
| | -3 | Vehicle[a] | 2.5 | 0 | 0 | 3 |
| JCP 10,000 JAU solution | -1 | JCP[b] | 0.1 | 1,000 | 250 | 3 |
| | -2 | JCP[b] | 0.5 | 5,000 | 1,250 | 3 |
| | -3 | JCP[b] | 2.5 | 25,000 | 6,250 | 3 |

[a] 50% Glycerin, 5% Sodium chloride
[b] Standardized JCP-allergen extract with allergen potency of 10,000 JAU/mL
[c] Calculated based on 250 g of rat body weight

### (4) Clinical observations, body weights and food consumption

All animals were observed twice daily (before dosing and after dosing) for clinical signs during the dosing period (from days 1 to 14). Body weights were

measured immediately before the initiation of dosing on day 1, 4, 8, 11 and on the last day of dosing (day 14). A feeding vessel containing food was weighed and set in the cage. The remaining diet was weighed at 24 h to calculate food consumption on 1, 4, 8, 11 and on the last day of dosing (day 14).

**(5) Hematology and blood chemistry**

Hematology was conducted using blood and plasma obtained at necropsy. The animals were fasted for 19–22 h before blood sampling. Ten hematological parameters were measured: numbers of leukocytes and erythrocytes, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, reticulocyte counts, platelets and differential leukocyte counts, using a hematology system (ADVIA 120, Siemens Healthcare Diagnostics, Deerfield, IL, USA). Two hematological parameters—prothrombin time and activated partial thromboplastin time—were determined by an autocoagulometer (Sysmex CA-5000, Sysmex Corporation, Kobe, Japan).

Blood chemistry was conducted using sera obtained at necropsy. Estimations of various biochemical parameters: total protein, total bilirubin, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total cholesterol, triglycerides, phospholipids, glucose, blood urea nitrogen, creatinine, inorganic phosphorus and Ca, were performed using an auto-analyzer (7170, Hitachi Ltd., Tokyo, Japan). Serum protein fractions (total protein × ratio), albumin and A/G ratio were estimated by an electrophoresis system (AES320, Beckman Coulter, Brea, CA, USA). Na, K and Cl were estimated by an electrolyte analyzer (EA07, A&T).

### (6) Necropsy, organ weights and histopathology

Following euthanasia by exsanguination from the abdominal inferior vena cava under pentobarbital anesthesia, all animals were examined in detail for gross lesions. Submandibular glands (with sublingual glands), liver, lungs (with bronchi), thymus, spleen, heart, kidneys, prostate gland, seminal vesicle, testes, epididymis, ovaries, uterus, pituitary, thyroids (with parathyroids), adrenals and brain were weighed after macroscopic examination, and the relative weight of each organ to the final body weight was calculated. For histopathology, after processing sections and staining with hematoxylin and eosin (HE) for light microscopy, the esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, and the organs and tissues above that were examined macroscopically were analyzed histopathologically in Vehicle-3 and JCP-3 groups. Organ weights were expressed as relative weights versus body weights (100 g) measured at necropsy day in all groups.

### (7) Statistical analysis

Statistical analysis was performed for body weight, food consumption, hematology, blood chemistry and organ weights using the MiTOX-PPL system (Mitsui Zosen Systems Research Inc.). For multiple comparisons, mean values and standard deviations were calculated for vehicle control group and treatment groups. For comparisons between two groups, mean values and standard deviations (SD) were calculated for each vehicle control groups. The homogeneity of variances was evaluated using the F-test (significance level: 5%). For homogeneous data, the Student’s t-test was used to compare with the vehicle control group. For heterogeneous data, Welch’s test was performed to compare with the control group. For comparison between the two groups (significance level: 5%), a two-sided test was used ($P < 0.05$, $P < 0.01$)

## 3.2 Results and Discussion

No test article related mortality was observed in any JCP-allergen extract treatment group and there were no treatment related changes in clinical observations, body weights (Figure 19) and food consumption (data not shown) during the 14-day dosing period.

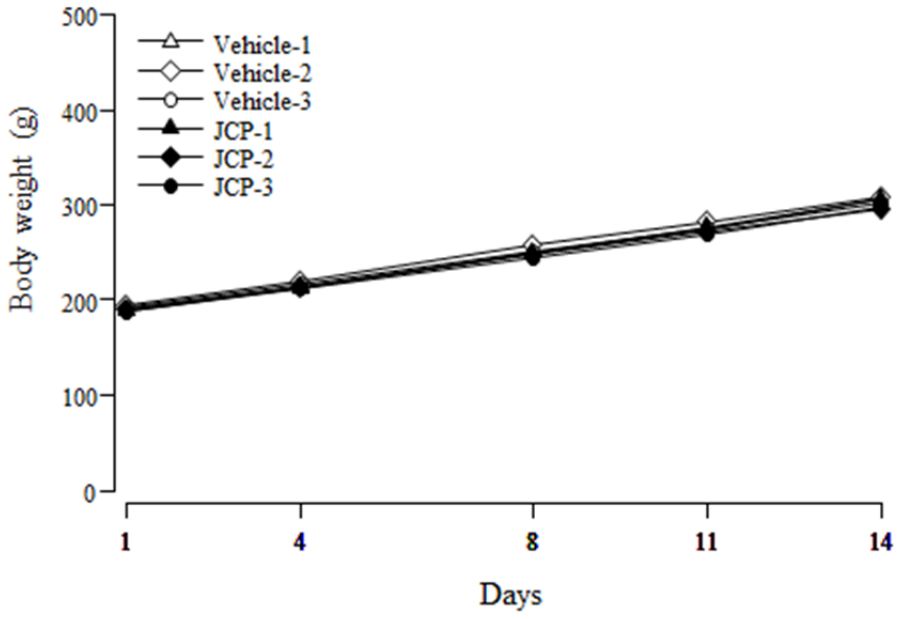



**Figure 19. Body weights of rats following oral administration of JCP-allergen extract.**

Rats were administered 200, 2,000 or 10,000 JAU/kg of JCP-allergen extract or vehicle (0 JAU/kg) daily for 26 weeks. Values are expressed as mean ± SD of 18 animals for all groups except for female control group where n=17 and 200 JAU/kg and 2,000 JAU/kg groups where n=12.

Results for hematology are shown in Table 11. Treatment-related changes were not observed in the JCP-allergen extract treatment group compared with the each vehicle control group.

**Table 11. Hematological finding of rats following 14 days oral administration of JCP-allergen extract.**

| | Vehicle | | | JCP | | |
|---|---|---|---|---|---|---|
| Dose (mL/kg) | -1 | -2 | -3 | -1 | -2 | -3 |
| Leukocytes ($10^3$/mL) | 9.06 ± 3.62 | 8.80 ± 0.21 | 8.35 ± 2.13 | 10.13 ± 2.15 | 8.01 ± 0.91 | 9.04 ± 1.45 |
| Erythrocytes ($10^4$/mL) | 712 ± 37 | 679 ± 6 | 707 ± 30 | 687 ± 57 | 715 ± 29 | 688 ± 25 |
| Hemoglobin (g/dL) | 14.5 ± 0.6 | 14.0 ± 0.5 | 14.4 ± 0.6 | 13.8 ± 0.6 | 14.2 ± 0.8 | 14.2 ± 0.4 |
| Hematocrit (%) | 42.9 ± 1.3 | 41.1 ± 1.3 | 42.5 ± 1.9 | 40.1 ± 1.8 | 41.9 ± 2.2 | 41.5 ± 0.8 |
| Mean corpuscular volume (fL) | 60.3 ± 1.4 | 60.5 ± 1.4 | 60.1 ± 0.2 | 58.6 ± 2.7 | 58.5 ± 1.6 | 60.3 ± 1.9 |
| Mean corpuscular hemoglobin:MCH (pg) | 20.4 ± 0.2 | 20.6 ± 0.6 | 20.4 ± 0.2 | 20.2 ± 1.1 | 19.9 ± 0.7 | 20.5 ± 0.3 |
| MCH concentration (g/dL) | 33.8 ± 0.5 | 34.0 ± 0.6 | 33.9 ± 0.4 | 34.4 ± 0.2 | 34.0 ± 0.3 | 34.1 ± 0.7 |
| Reticulocyte ($10^4$/mL) | 22.0 ± 2.9 | 21.1 ± 1.4 | 22.8 ± 2.0 | 24.3 ± 2.0 | 18.8 ± 6.7 | 21.8 ± 0.7 |
| Platelets ($10^4$/mL) | 124.4 ± 1.7 | 128.7 ± 10.0 | 123.3 ± 11.3 | 117.9 ± 2.1* | 111.7 ± 10.7 | 122.9 ± 19.1 |
| Prothrombin time (sec) | 13.9 ± 0.9 | 14.5 ± 0.7 | 15.0 ± 2.0 | 13.2 ± 1.1 | 16.0 ± 2.5 | 13.6 ± 0.7 |
| Activated partial thromboplasmin time (sec) | 21.9 ± 0.5 | 21.4 ± 1.6 | 23.1 ± 2.1 | 20.1 ± 1.4 | 22.1 ± 1.1 | 20.9 ± 1.2 |
| Eosinophils ($10^3$/mL) | 0.05 ± 0.01 | 0.05 ± 0.01 | 0.05 ± 0.01 | 0.06 ± 0.03 | 0.06 ± 0.01 | 0.12 ± 0.01** |
| Neutrophils ($10^3$/mL) | 1.35 ± 0.35 | 1.37 ± 0.34 | 1.30 ± 0.31 | 1.67 ± 0.53 | 1.17 ± 0.18 | 1.44 ± 0.46 |
| Lymphocytes ($10^3$/mL) | 7.44 ± 3.23 | 7.13 ± 0.50 | 6.72 ± 1.67 | 8.13 ± 2.35 | 6.49 ± 0.98 | 7.23 ± 1.00 |
| Basophil ($10^3$/mL) | 0.03 ± 0.02 | 0.03 ± 0.01 | 0.02 ± 0.01 | 0.03 ± 0.01 | 0.02 ± 0.00 | 0.03 ± 0.01 |
| Monocytes ($10^3$/mL) | 0.14 ± 0.02 | 0.18 ± 0.04 | 0.22 ± 0.12 | 0.18 ± 0.04 | 0.20 ± 0.03 | 0.17 ± 0.01 |
| Large unstained cells ($10^3$/mL) | 0.05 ± 0.01 | 0.05 ± 0.00 | 0.05 ± 0.03 | 0.08 ± 0.02 | 0.06 ± 0.02 | 0.06 ± 0.01 |

Values are expressed as mean ± SD of 3 animals in each group.
*: $p<0.05$, **: $p<0.01$ (significantly different from 0 JAU/kg).

Results for blood chemistry are shown in Table 12. Treatment-related changes were not observed in JCP treatment groups compared with the each vehicle control group.

**Table 12. Blood chemical finding of rats following 14 days oral administration of allergen extract of Japanese cedar pollen.**

| | Vehicle | | | JCP | | |
|---|---|---|---|---|---|---|
| Dose (mL/kg) | -1 | -2 | -3 | -1 | -2 | -3 |
| Total protein (g/dL) | 5.0 ±0.1 | 5.0 ±0.2 | 4.9 ±0.2 | 4.9 ±0.2 | 4.9 ±0.1 | 5.2 ±0.2 |
| Albumin (g/dL) | 2.68 ±0.10 | 2.73 ±0.12 | 2.72 ±0.13 | 2.84 ±0.05 | 2.79 ±0.01 | 2.94 ±0.06 |
| A/G ratio | 1.17 ±0.07 | 1.21 ±0.02 | 1.24 ±0.03 | 1.36 ±0.08* | 1.30 ±0.04* | 1.33 ±0.16 |
| a1-Globulin (%) | 19.8 ±0.9 | 20.6 ±0.7 | 20.6 ±1.0 | 18.4 ±2.5 | 17.7 ±2.3 | 18.5 ±2.9 |
| a2-Globulin (%) | 7.7 ±0.5 | 6.9 ±0.6 | 6.3 ±0.7 | 7.0 ±0.5 | 7.1 ±0.4 | 6.9 ±0.7 |
| b-Globulin (%) | 14.4 ±0.8 | 13.9 ±0.3 | 13.7 ±0.1 | 13.3 ±1.1 | 14.8 ±0.9 | 13.5 ±0.9 |
| g-Globulin (%) | 4.2 ±1.0 | 4.1 ±0.4 | 4.0 ±0.6 | 3.7 ±0.4 | 4.0 ±0.9 | 4.1 ±1.2 |
| Albumin (%) | 53.9 ±1.3 | 54.6 ±0.3 | 55.4 ±0.7 | 57.6 ±1.4* | 56.5 ±0.8 | 57.0 ±2.9 |
| Total bilirubin (mg/dL) | 0.0 ±0.0 | 0.0 ±0.0 | 0.0 ±0.0 | 0.0 ±0.0 | 0.0 ±0.0 | 0.0 ±0.0 |
| Asparate aminotransferase (IU/L) | 76 ±4 | 90 ±6 | 82 ±7 | 70 ±9 | 80 ±16 | 68 ±2* |
| Alanine aminotransferase (IU/L) | 22 ±5 | 19 ±1 | 22 ±1 | 19 ±2 | 18 ±3 | 19 ±2 |
| Alkaline phosphatase (IU/L) | 511 ±98 | 580 ±75 | 496 ±28 | 517 ±131 | 482 ±71 | 494 ±55 |
| Total cholesterol (mg/dL) | 58 ±2 | 70±10 | 61±22 | 85 ±16 | 69±11 | 75±12 |
| Triglycerides (mg/dL) | 41 ±14 | 54 ±16 | 59 ±23 | 60 ±2 | 60 ±22 | 95 ±12 |
| Phospholipids (mg/dL) | 109 ±2 | 120 ±14 | 111 ±25 | 140 ±16 | 125 ±11 | 138 ±13 |
| Glucose (mg/dL) | 120±4 | 106±14 | 101±10 | 119±10 | 120±17 | 116±10 |
| Blood urea nitrogen (mg/dL) | 15.1 ±0.5 | 14.4 ±1.1 | 12.2 ±1.5 | 15.1 ±02.4 | 11.2 ±0.7* | 13.6 ±1.7 |
| Creatinine (mg/dL) | 0.4 ±0.0 | 0.3 ±0.1 | 0.4 ±0.0 | 0.4 ±0.0 | 0.3 ±0.1 | 0.4 ±0.1 |
| Inorganic phosphorus (mg/dL) | 9.1 ±0.6 | 8.9 ±0.5 | 9.0 ±0.3 | 9.6 ±0.6 | 9.1 ±0.3 | 9.0 ±0.2 |
| Ca | 10.0 ±0.1 | 10.2 ±0.2 | 10.0 ±0.4 | 10.0 ±0.5 | 10.1 ±0.3 | 10.4 ±0.2 |
| Na | 146.6 ±0.3 | 146.7 ±0.3 | 146.6 ±0.6 | 146.3 ±1.4 | 146.1 ±0.7 | 146.6 ±1.1 |
| K | 4.28 ±0.07 | 4.25 ±0.21 | 4.33 ±0.34 | 4.16 ±0.10 | 4.22 ±0.20 | 4.27 ±0.20 |
| Cl | 105.0 ±0.7 | 104.8 ±1.1 | 105.1 ±0.6 | 103.8 ±1.0 | 104.4 ±1.5 | 103.6 ±0.5* |

Values are expressed as mean ± SD of 3 animals in each group.
*: $p<0.05$ (significantly different from 0 JAU/kg).

No gross abnormalities were detected for rats in any group by necropsy at the treatment period and recovery period (data not shown).

There were no treatment-related differences in organ weights between the vehicle control and 10,000 JAU/kg groups (Figure 20).

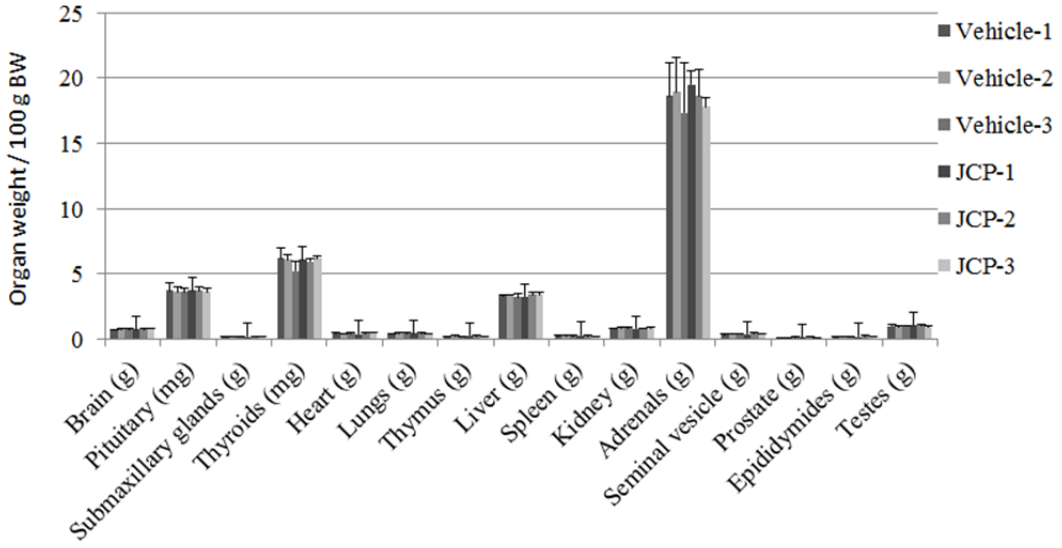



**Figure 20. Organ weights of rats following 26 weeks oral administration of JCP-allergen extract.**

Rats were orally administered 250, 1,250 and 6,250 JAU/head of JCP-allergen extract (JCP-1, JCP-2. JCP-3) or vehicle (0 JAU/head; Vehicle-1, Vehicle-2, Vehicle-3) daily for 14 days at each dosage volume. Values are expressed as mean±SD of 3 animals in each group.

Results for histopathology are shown in summarized Table (Table 13). Histopathology revealed no toxicological changes in the mucosa of GI tract of the animals from vehicle -3 and JCP-3 groups (Figure 21) although some changes in organs occurred sporadically. In kidney, the pyelectasis were observed in each one animal of vehicle-3 and JCP-3 group (Table 13). They were slight changes and it is known that such changes are observed occasionally in normal animals.

**Table 13. Histopathological findings of rats following 14days oral administration of JCP-allergen extract.**

| Finding | Dose | |
|---|---|---|
| | Vehicle-3 (0 JAU/site) | JCP-3 (6,250 JAU/site) |
| Digestive system | | |
| Esophagus | 0 | 0 |
| Stomach | 0 | 0 |
| Duodenum | 0 | 0 |
| Jejunum | 0 | 0 |
| Ileum | 0 | 0 |
| Cecum | 0 | 0 |
| Colon | 0 | 0 |
| Rectum | 0 | 0 |
| Liver | 0 | 0 |
| Urinary system | | |
| Kidney | 0 | 0 |
| Dilatation, pelvic cavity | 1 | 1 |
| Endocrine system | | |
| Adrenal | 0 | 0 |

Three rats/group were administered 2.5 mL of 10,000 JAU/mL JCP-allergen extract (JCP-3; 6,250 JAU/site) or vehicle (Vehicle-3; 0 JAU/site) orally for 14 days.

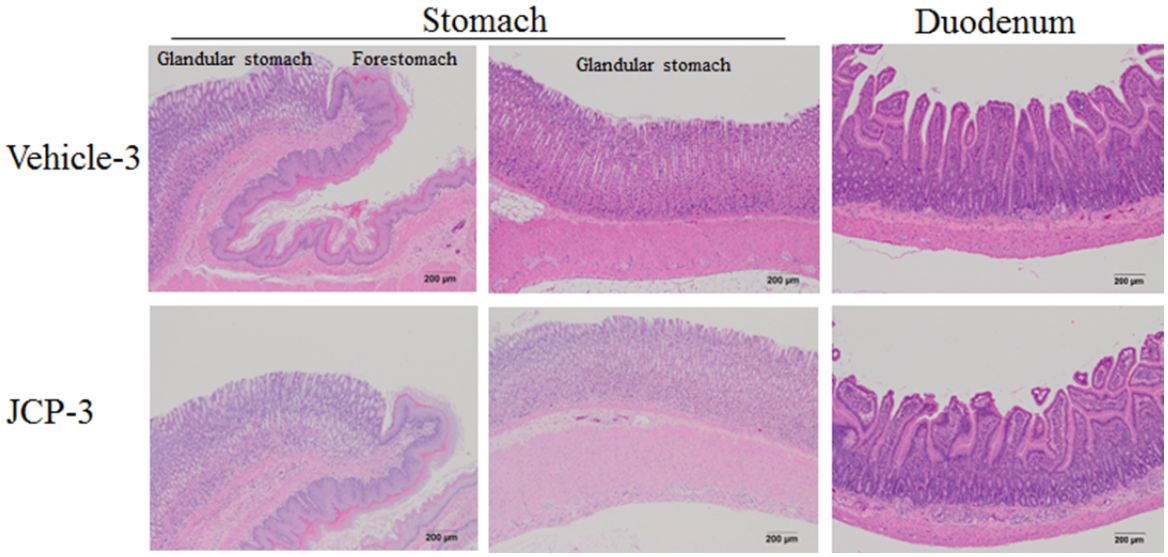



**Figure 21. H&E staining of GI mucosa sections from rats following 14 days oral administered of JCP-allergen extract.**

Three rats/group were administered 2.5 mL of 10,000 JAU/mL JCP-allergen extract (JCP-3; 6,250 JAU/site) or vehicle (Vehicle-3; 0 JAU/site) orally for 14 days. The bars indicate 200 µm.

### 3.3 Short Summary

The present findings show neither irritation of the GI tract mucosa nor systemic toxicity in rats after oral treatment with a JCP-allergen extract. There were no treatment-related changes on the observation on necropsy in the GI tract and by histopathology of mucosal tissues of esophagus, stomach, duodenum, jejunum, ileum, cecum, colon and rectum. It suggested no local irritation of the GI mucosa occurred in healthy rats after 14 days repeated oral treatment with JCP-allergen extract.

The doses used in the study were much higher in systemic exposure (~800-fold based on 60 kg of human body weight) compared to the doses used in clinical, suggesting that SLIT-swallow at clinical doses should not exert remarkable adverse effects in patients with JC pollinosis.

## 4 Summary

Two studies on local irritation were conducted as safety assessment of standardized JCP-allergen extract currently developed for SLIT in JC pollinosis. Oral mucosa was examined in the rabbits as first-of-contact site for SLIT. Mucosa of GI tract was examined in the rats as the contact site after swallowing of allergen extract in SLIT.

Regarding oral mucosa, no treatment-related changes was seen in macroscopic observation throughout the dosing period and by histopathology of oral mucosal tissues including tongue, buccal and mouth floor. It suggested no local irritation of the sublingual/buccal mucosa occurred in healthy rabbits after 7 days repeated sublingual treatment with JCP-allergen extract drug product with 10-times longer application than for clinical use.

Regarding GI tract mucosa, no treatment-related changes in observation of the GI tract on necropsy on last day in 14 days dosing period and by histopathology of mucosal tissues of GI tract including the esophagus, stomach, duodenum, jejunum, ileum, cecum, colon and rectum. The doses used in the studies were much higher in systemic exposure based on body weight (~800-fold, 60 kg as human body weight) and more than 3-fold (6,250 JAU in rats by po vs 2,000 JAU in human by sublingual administration) in local exposure compared with the doses used in clinical. Local exposure level in this study is considered to be sufficient high compared to clinical use because actually a part of 2,000 JAU reached to GI tract in human such as stomach after swallowing in SLIT resulting much higher safety margin than 3-fold.

These data suggest that SLIT-swallow at clinical doses should not exert remarkable adverse effects in patients with JC pollinosis although further study is required in JCP extract -sensitized animals to evaluate the local irritation that appeared during SLIT in patients with JC pollinosis.

# Chapter V. SAFETY EVALUATION ON GENOTOXICITY OF ALLERGEN EXTRACT OF JAPANESE CEDAR POLLEN

## 1 Introduction

Safety evaluation on genotoxicity is important for the development of new drug to assess the risk of carcinogenicity. For the new drug application to authority, it is required to extensive evaluation of genotoxicity with several tests and scientific explanation on genotoxicological risk of developmental product based on the data obtained from *in vitro* and *in vivo* tests (PFSB/ELD Notification 0920 No. 2, dated September 20, 2012). The ICH harmonized guideline among Japan, Europe and US indicates the standard battery of genotoxicity (ICH S2(R1), dated 9 November 2011).

The bacterial reverse gene mutation test (also referred to as Ames test) is test system for the assessment of mutagenicity with detection of relevant genetic changes and widely used for screening from the point of view of genotoxicity and carcinogenicity. The majority of genotoxic rodent and human carcinogens are detected by this test. In this test system, auxotrophic strains of bacteria are used which caused mutation in the genes involved in amino acid metabolism. It is possible to count the forming colonies in medium amino acid is insufficient when reverse mutation occurs. Potentials of chromosomal aberration and micronuclei induction could be evaluated in mammalian cells *in vitro* and *in vivo*

Regarding the extensive review following to the guideline, a series of genotoxicity study was conducted for the safety evaluation on genotoxicity of JCP; reverse mutation test in bacterial cells (Ames test), *in vitro* chromosomal aberration test and *in vivo* bone marrow micronucleus test (Table 14).

**Table 14. The battery of geontoxicity study of JCP-allergen extract.**

| ICH guideline S2(R1) Option 1 | Categories of the mutagenicity tests | Materials | Test for JCP |
|---|---|---|---|
| **i.**Test for gene mutation in bacteria (Ames test) | Gene mutation | Bacteria | Reverse mutation test in bacterial cells (Ames test) |
| **ii.***In vitro* Cytogenetic test for chromosomal damage | Chromosome aberration | Mammalian cells | *In vitro* chromosomal aberration test in Chinese hamster lung cells |
| **iii**.*In vivo* test for genotoxicity | Chromosomal damage using rodent hematopoietic cells | Animal | *In vivo* bone marrow micronucleus test in rats |

All studies were conducted in accordance with the Guidelines for Genotoxicity Studies in compliance with the GLP Regulations specified by the Japanese Ordinance.

## 2 Materials and Methods

### 2.1 Standardized allergen extract of Japanese cedar pollen

Standardized JCP-allergen extract is glycerinated JCP extract obtained by managed process and controlled by the content of Cry j 1, a major allergen protein of Japanese cedar pollen (Yasueda et al., 1983), and its allergen potency is expressed as Japanese allergen unit (JAU) defined based on skin reaction (Yasueda et al., 1996). Standardized JCP-allergen extract original solution 10,000 JAU/mL (10,000 JAU/mL JCP) is defined to contain 7.3-21 ug/mL of Cry j 1(Yasueda et al., 1996). Test liquids of 200 and 2,000 JAU/mL were formulated by dilution of 10,000 JAU/mL JCP to provide the indicated potency, respectively (Torii Pharmaceutical Co. Ltd., Tokyo, Japan).

In genotocixity study (bacterial reverse mutation test; that is Ames test, *in vitro* chromosomal aberration test and *in vivo* micronucleus test), 10,000 JAU/mL JCP and its diluted solutions were used. Doses of 12,500, 25,000 and 50,000 JAU/kg were administered subcutaneously to rats in bone marrow micronucleus test. In addition, 50% glycerin-containing sodium chloride was used for the vehicle control group.

### 2.2 Reverse mutation test in bacterial cells (Ames test)

The mutagenic potential of JCP was evaluated in a bacterial reverse mutation test using four histamine auxotrophic strains of Salmonella typhimurium (S. typhimurium) TA98, TA100, TA1535 and TA1537 and one tryptophan auxotrophic strain of Escherichia coli (E. coli) WP2*uvrA*. The test solutions were prepared by dilution of 10,000 JAU/mL JCP, with 50% glycerin containing sodium chloride and treated using the pre-incubation method with or without metabolic activation with S9 mix (Aroclor$^{TM}$ 1254-induced rat liver S9,

Oriental Yeast Co. Ltd., Japan). The dose was expressed as %concentration of test solution. Two plates per treatment were used in the dose-range finding test and main test. The plates were incubated at 37˚C for 48 hours and the revertant colonies were counted and examined. The results were judged positive if the mean number of revertant colonies showed a dose dependent increase that was two-fold greater than that of vehicle control at one or more concentrations.

A dose-range finding test was performed on all of strains both with and without S9 mix at the following doses of 10,000 JAU/mL JCP. The 100% of 10,000 JAU/mL was selected as the highest dose and lower dose levels, 25.0, 6.25, 1.56, 0.391, 0.0977 and 0.0244% of JCP were set at a geometric ratio of 4. Distilled water was used as a negative control. The 50% glycerin solution containing sodium chloride was used as a vehicle control. Positive controls consisted of 0.01 μg/plate of 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (AF-2) for TA100 and WP2*uvrA* without S9 mix activation; 0.1 μg/plate of AF-2 for TA98 without S9 mix activation; 0.5 μg/plate of sodium-azide for TA1535 without S9 mix activation; 80 μg/plate of 9-aminoacridine hydrochloride for TA1537 without S9 mix activation; 1, 2, 10, 0.5, and 2 μg/plate of 2-aminoanthracene (2AA) for TA100, TA1535, WP2*uvrA*, TA98, and TA1537, respectively with S9 mix activation.

### 2.3 *In vitro* Chromosomal aberration test in Chinese hamster lung cells

The potential for JCP to induce chromosomal aberrations in cultured mammalian cells was evaluated using a fibroblast cell line from the lung of a Chinese hamster (CHL/IU; DS Pharma Biomedical, Osaka, Japan), either with or without S9 mix activation. The test solutions were prepared by dilution of standardized JCP-allergen extract original solution (10,000 JAU/mL) with

distilled water and treated with or without metabolic activation with S9 mix (Aroclor™ 1254-induced rat liver S9, Oriental Yeast). The dose was expressed as %concentration of test solution.

For short-time treatments, the treatment time was 6 hours and viable cell counts and preparation of chromosome specimens were conducted 18 hours after completion of treatment. For continuous treatment, the treatment time was 24 hours and viable cell counts and preparation of chromosome specimens were conducted at completion of treatment. Two dishes were used per dose, condition (with or without S9 mix activation) and exposure time. The positive control articles were diluted with culture media to dimethylnitrosamine (DMN; Wako Pure Chemical Industries, Osaka, Japan) set at a concentration of 500 μg/mL for the 6 and 24-h treatments without S9 mix activation and with mitomycin C (MMC; Kyowa Hakko Kirin, Tokyo, Japan) at a concentration of 0.1 μg/mL for the 6 and 24-h treatment without S9 mix activation. One hundred well-spread metaphases per dish were examined microscopically for structural and numerical chromosomal aberrations. The value of the incidence of cells with chromosomal aberrations was recorded according to the dish and the mean for each dose level was calculated. For each dose level, the mean incidences were evaluated as lower than 5%: negative, 5% or more but less than 10%: equivocal and 10% or more: positive, with no statistical comparisons being made.

Based from a dose-range finding study (dose-levels: 12.5, 25, 50 and 100% of vehicle; 50% glycerin containing sodium chloride), the dose level of the definitive test were selected as 6.25, 12.5 and 25% of the 10,000 JAU/mL JCP-allergen extract for the 6-h treatment with or without S9 mix activation and 24-h treatment without S9 mix activation. Thus, the final doses of JCP-allergen extract in the definitive test were 625, 1,250 and 2,500 JAU/mL.

## 2.4 *In vivo* Bone marrow micronucleus test in rats

An *in vivo* mammalian erythrocyte micronucleus test was conducted with approval of the Institutional Animal Care and Use Committee of Nihon Bioresearch Center, Japan in accordance with laboratory animal welfare guidelines.

Male Crl:CD (Sprague-Dawley) rats (Charles River, Japan), aged 5 weeks, with body weights of 135–148 g for a preliminary dose-finding study and 123–146 g for the definitive micronucleus study were housed in a 12-h light-dark cycle at a temperature of 21.7–24.5 ˚C, with *ad libitum* access to food (certified diet CRF-1, Oriental Yeast Co., Ltd.) and water. The 31 rats were stratified by body weight and randomly assigned to one of six groups (five rats/group) on the first day (day 1) prior to dosing. In the preliminary dose-finding study using doses of 12.5, 25, 50 and 100% JCP, no cytotoxicity was observed for all doses. The 100% dose was set as the highest dose, and doses of 50 and 25% were selected as middle and low doses, respectively. Animals were dosed twice subcutaneously with 5 mL/kg at 24 h-intervals. The doses of JCP-allergen extract were 12,500, 25,000 and 50,000 JAU/kg. A 50% glycerin solution containing sodium chloride and distilled water were administered subcutaneously as vehicle control and negative control, respectively. Mytomicin C (MMC, Kyowa Hakko Kirin) was used as a positive control and administered at a dose of 2 mg/kg/day by intraperitoneal administration.

All animals were observed for clinical signs, mortality and body weights were measured prior to and after dosing on days 1 and 2, and just prior the preparation of specimens. All animals were euthanized by 20 v/v% isoflurane anesthesia exposure 24 h after the final dosing. The femur was excised for the preparation of bone marrow cell suspensions. Slides coated with acridine orange were prepared for incidence of micronucleated polychromatic erythrocyte

(%MNPCE) analysis among 2,000 polychromatic erythrocytes per animal. To assess bone marrow cell proliferation, the ratio of reticulocytes (%RET) in 1,000 erythrocytes was analyzed using slides coated with new methylene blue for Giemsa staining.

Statistical analyses were performed between the negative control group and vehicle control group, negative control group and positive control group, and between vehicle control group and test article treated groups. The conditional binomial test (Kastenbaum–Bowman test) was used to calculate %MNPCE and the Student's t-test was to calculate %RET.

## 3 Results and Discussion

### 3.1 Reverse mutation test in bacterial cells (Ames test)

In the dose-finding test, inhibition of bacterial growth was observed on the plates at 100% of JCP for TA1537 without S9 mix. However no inhibition of bacterial growth inhibition was observed at any dose tested for TA98. TA100, TA1535, TA1537 and WP2*uvrA* with or without S9 mix and TA 1537 with S9 mix. No precipitation of the test article was observed at the start or end of the treatment in all conditions. The test article did not increase the number of revertant colonies of any strain ≥ two-fold when compared with the vehicle control with or without S9 mix (data not shown). From these results, the definitive test was performed using the test solutions of 3.125, 6.25, 12.5, 25, 50 and 100% of JCP-allergen extract on all strains without S9 mix.

In the definitive test (Table 15), inhibition of bacterial growth was observed on the plates at 1,000 JAU/plate of JCP-allergen extract for TA1537 without S9 mix. However, no inhibition of bacterial growth inhibition was observed at any dose tested for TA98, TA100, TA1535 and WP2*uvrA* with or without S9 mix and TA1537 with S9 mix. The test article did not increase the number of revertant colonies of any strain by ≥two-fold compared with the vehicle control with or without S9 mix (Table 2). No precipitation of the test article was observed either at the start or end of the treatments under all the conditions.

**Table 15. Results of bacterial reverse mutation test (Ames test) conducted on JCP-allergen extract.**

| Concentration (JAU/plate) | Reverse colonies per plate (mean ± SD ) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | TA100 | | TA1535 | | WP2*uvrA* | | TA98 | | TA1537 | |
| | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 |
| Negative control (distilled water) | 138 | 142 | 11 | 7 | 31 | 40 | 19 | 32 | 14 | 19 |
| 0 | 151 | 143 | 11 | 8 | 32 | 32 | 18 | 35 | 10 | 20 |
| 31.25 | NT | 154 | NT | 9 | NT | 26 | NT | 34 | 7 | 18 |
| 62.5 | 147 | 154 | 12 | 7 | 29 | 41 | 21 | 26 | 11 | 16 |
| 125 | 131 | 144 | 8 | 8 | 37 | 37 | 19 | 29 | 8 | 17 |
| 250 | 166 | 149 | 10 | 7 | 31 | 36 | 18 | 31 | 11 | 20 |
| 500 | 139 | 162 | 10 | 8 | 22 | 29 | 19 | 38 | 12 | 17 |
| 1,000 | 156 | NT | 9 | NT | 36 | NT | 13 | NT | 10[c] | NT |
| Positive controls [a, b] | 538 | 1,068 | 478 | 283 | 132 | 1,092 | 481 | 380 | 401 | 191 |

NT: not tested
[a] positive controls –S9: 0.01 µg/plate 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (AF-2) for TA100 and WP2*uvrA*, 0.1 µg/plate AF-2 for TA98, 0.5 µg/plate sodium-azide for TA1535, 80 µg/plate 9-aminoacridine hydrochloride for TA1537.
[b] positive controls +S9: 0.5 1 and 10 µg/plate 2-aminoanthracene (2AA) for TA98, TA100 and WP2*uvrA* , respectively. 2 µg/plate 2AA for TA1535 and TA1537.
[c] Bacterial growth inhibition was observed.

### 3.2 *In vitro* Chromosomal aberration test in Chinese hamster lung cells

The dose-finding test of the chromosomal aberration test was conducted with vehicle (50% glycerin containing sodium chloride) at doses of 12.5, 25, 50 and 100%. The incidence of cells with numerical chromosomal aberrations was less than 5% under all conditions. The 50 and 100% vehicle in the short treatment without S9 mix was equivocal and 100% of vehicle for continuous treatment was positive for the incidence of structural chromosomal aberrations of 5, 8 and 16%, respectively (data not shown). Cell viability was >90% with no cytotoxicity

under all conditions in the dose-finding test. No precipitation of the test article was observed at the start or end of treatments under all the conditions in the dose-finding test.

In the definitive test, test solutions were 6.25, 12.5 and 25% of JCP-allergen extract, thus the final doses of treatment were 625, 1,250 and 2,500 JAU/mL for the 6-h treatment with or without S9 mix and 24-h treatment without S9 mix. The incidence of cells with numerical and structural chromosomal aberrations was less than 5% in all JCP treatment group with short treatment or continuous treatment (Table 16). The negative control induced no chromosomal aberrations as expected. The positive control agents increased the percentage of cells with numerical or structural chromosomal aberrations. Thus, JCP-allergen extract was not considered clastogenic in this test system. No precipitation of the test article was observed at the start or end of the treatments in all the conditions.

**Table 16. Results of *in vitro* mammalian chromosomal aberration test conducted on JCP-allergen extract.**

| Concentration (JAU/mL) | Numerical aberration | Structural aberration | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Number of polyploid cells (%) | Types and numbers (cumulative) [a] | | | | | | Number of cells with chromosomal aberration (%) [b] | |
| | | gap | ctb | csb | cte | cse | frg | +g | -g |
| Short-time treatment, -S9 | | | | | | | | | |
| Negative control (distilled water) | 1 (0.5) | 0 | 0 | 0 | 1 | 0 | 0 | 1 (0.5) | 1 (0.5) |
| 625 | 2(1.0) | 0 | 1 | 0 | 1 | 1 | 0 | 3 (1.5) | 3 (1.5) |
| 1,250 | 3 (1.5) | 0 | 1 | 0 | 0 | 0 | 0 | 1 (0.5) | 1 (0.5) |
| 2,500 | 1 (0.5) | 0 | 0 | 0 | 0 | 0 | 0 | 0 (0) | 0 (0) |
| Positive control [c] | 1 (0.5) | 0 | 63 | 2 | 69 | 1 | 0 | 108 (54.0) | 108 (54.0) |
| Short-time treatment, +S9 | | | | | | | | | |
| Negative control (distilled water) | 1 (0.5) | 0 | 1 | 0 | 1 | 0 | 0 | 2 (1.0) | 2 (1.0) |
| 625 | 2 (1.0) | 0 | 1 | 0 | 1 | 0 | 0 | 2 (1.0) | 2 (1.0) |
| 1,250 | 1 (0.5) | 0 | 0 | 0 | 0 | 0 | 0 | 0 (0) | 0 (0) |
| 2,500 | 3 (1.5) | 0 | 0 | 0 | 0 | 0 | 0 | 0 (0) | 0 (0) |
| Positive control [d] | 1 (0.5) | 0 | 52 | 2 | 85 | 0 | 0 | 115 (57.5) | 115 (57.5) |
| Continuous treatment | | | | | | | | | |
| Negative control (distilled water) | 1 (0.5) | 0 | 2 | 0 | 0 | 0 | 0 | 2 (1.0) | 2 (1.0) |
| 625 | 0 (0) | 0 | 0 | 0 | 0 | 0 | 0 | 0 (0) | 0 (0) |
| 1,250 | 2 (1.0) | 0 | 0 | 0 | 0 | 0 | 0 | 0 (0) | 0 (0) |
| 2,500 | 2 (1.0) | 0 | 0 | 0 | 1 | 0 | 0 | 1 (0.5) | 1 (0.5) |
| Positive control [e] | 0 (0) | 0 | 45 | 2 | 36 | 0 | 0 | 81 (40.5) | 81 (40.5) |

−S9, with metabolic activation; +S9, with metabolic activation; JAU, Japanese allergy units; a: ctb, chromatid break; csb, chromosome break; cte: chromatid exchange; cse: chromosome exchange; frg, fragmentation; b: (+g): total aberrant cells including the gap; (−g): total aberrant cells excluding the gap; c: 0.1 μg.mL mitomycin C; d: 500 μg/mL dimethylnitrosamine; e: 0.05 μg.mL mitomycin C.

### 3.3 *In vivo* Bone marrow micronucleus test in rats

In the *in vivo* micronucleus test, no mortality was observed following two administrations of JCP-allergen extract at 12,500, 25,000 or 50,000 JAU/kg. No clinical signs or change in body weight was observed when compared with the vehicle control group. The incidence of MNPCE and ratio of RET are shown in Table 17.

The %MNPCE in the JCP treatment group showed no significant difference compared with the vehicle control group and no dose-dependent change. No significant difference was observed for %RET compared with the vehicle control group. Administration of the positive control significantly increased the incidence of micronucleated cells as MNPCE and resulted in a significant decrease in the ratio of immature erythrocytes as RET compared with the negative control group. There was no possibility of the vehicle having an effect on the test system because there was no significant difference in the incidence of MNPCE and %RET compared with the negative control. Thus, it was concluded that JCP-allergen extract did not induce micronuclei in PCE of bone marrow of rats treated with up to 50,000 JAU/kg by subcutaneous administration.

**Table 17. Results of *in vivo* mammalian erythrocyte micronucleus test in rats administered with JCP-allergen extract.**

| Dosage (JAU/kg) | %MNPCE | %RET |
|---|---|---|
| Negative control (distilled water) | 0.09 ± 0.04 | 76.7 ± 0.54 |
| 0 | 0.08 ± 0.03 | 76.1 ± 0.84 |
| 12,500 | 0.12 ± 0.07 | 76.3 ± 0.97 |
| 25,000 | 0.13 ± 0.04 | 76.8 ± 0.95 |
| 50,000 | 0.07 ± 0.06 | 76.7 ± 2.30 |
| Positive control (2 mg/kg mitomycin C) | 3.40 ± 0.34** | 66.3 ± 3.02## |

Animals were dosed twice with JCP-allergen extract and negative control at a 24h-interval subcutaneously.
Positive control was dosed by intraperitoneal administration.
Values are expressed as mean ± SD of 5 animals in each group.
**: $p<0.01$ (significantly different from negative control).
##: $p<0.01$ (significantly different from negative control).

Regarding the safety margin, highest dose of this study, 50,000 JAU/kg/day, corresponds to approximately 1,500-times higher than the intended clinical dose level (2,000 JAU/man/day by sublingual administration) based on body weight. Thus, it is suggested that the JCP does not pose the potential to induce micronuclei for clinical use. This is supported by the absence of any adverse genotoxic or carcinogenic effects during over 15 years of clinical experience with SCIT products with Japanese cedar pollen allergen extracts.

## 4 Summary

The safety evaluation on genotoxicity of JCP was conducted by a series of two *in vitro* tests and one *in vivo* test. The *in vitro* tests were reverse mutation test in bacterial cells (Ames test) to assess the mutagenic properties and mammalian chromosomal aberration test in Chinese hamster lung cells to evaluate the potential for induction structural or numerical chromosome aberrations. The *in vivo* test was mammalian erythrocyte micronucleus test in rats to determine the potential to induce micronuclei.

The Ames test detected no cytotoxic effects except for TA1537 at 100% JCP without S9 mix, or mutagenic effect. The chromosomal aberration test was conducted at highest concentration of 25%JCP as non-effective dose for the test system, and showed that JCP induced no numerical or structural aberrations and was not clastogenic. In addition, the *in vivo* micronucleus test showed no increase in micronuclei frequency in polychromatic erythrocytes. Regarding the safety margin of *in vivo* micronucleus test, highest dose of this study, 50,000 JAU/kg/day, corresponds to approximately 1,500-times higher than the intended clinical dose level (2,000 JAU/man/day by sublingual administration) based on body weight.

In this study, I presented that the genotoxicity potential in clinical use is considered to be very low. This is supported by the absence of any adverse genotoxic or carcinogenic effects during over 15 years of clinical experience of Japanese cedar pollen allergen extracts as SCIT products.

# Chapter VI. CONCLUSION AND PERSPECTIVE

Prevalence of JC pollinosis have been increasing (Kaneko et al, 2004; Okubo et al., 2011; Yamada et al., 2014) and it is considered to be a national affliction (Kaneko et al., 2005; Yamada et al., 2014) in Japan. Generally in allergic disease, the first approach to deal with is avoidance of antigens/allergen such as pets (eg. dogs and cats) and some foods if it is possible. It is difficult to completely eliminate antigens/allergen such as house dust mite and pollen though they could be reduced by cleaning and dehumidification for house dust mite and taking a mask and glasses is effective for pollen. Several type of treatment are recommended against allergic rhinitis in the Japanese Guideline for allergic rhinitis (Okubo et al., 2011); pharmacotherapy (chemical mediator receptor antagonist such as anti-histamine, mast cell stabilizer, steroids, autonomic drugs and others), operative treatment and AIT including SCIT and SLIT. Recently, SLIT has much attention and considered to be a safe and efficient treatment as being possible to cure allergic disease although symptomatic treatment by pharmacotherapy allows tentative relief from symptoms (Bousquet et al., 1998; Didier et al., 2011; Nelson et al., 2011; Cox et al., 2012; Durham et al., 2012).

Here, safety was evaluated for the development of a drug for SLIT with standardized JCP-allergen extract (Sublingual droplet of JCP). The JCP-allergen extract exhibited safe profile in long-term repeated toxicity study, local irritation study (oral area, GI tract) and *in vitr*o and *in vivo* genotoxicity studies, suggesting it is suitable for SLIT.

In **Chapter I**, introduction and background information was noted regarding JC pollinosis caused by JCP, allergen extract of JCP, AIT including

SCIT and SLIT, standardization of allergen extract for AIT, background of development of Sublingual droplet of JCP. In **Chapter II,** the biological profile of JCP-allergen extract was discussed. Levels of the JCP specific-antibody in serum was significantly increased following i.p. administration with alum compared with naïve serum, showing immunogenicity of JCP-allergen extract. Allergic specific immune reaction was confirmed by PCA reaction in rats, indicating allergen profile of JCP-allergen extract. These are basic information of biological profile showed by JCP-allergen extract which is considered to modulate immunoreactions known in the mechanism of AIT. In addition, it is very interesting that there was the difference of reactivity of JCP-allergen extract in saliva and gastric juice in *in vitro* assay. The activity of major allergens (Cry j 1 and Cry j 2) was detected in human saliva although disappeared immediately in gastric juice. It suggests that major allergens in JCP are stable in the mouth of JC pollinosis patients, and immediately metabolized/degraded following been swallowed and reached to stomach. The missing activity of proteins in gastric juice is considered to be consistent with less incidence of systemic adverse effect seen in SLIT-swallow.

In **Chapter III**, no JCP-allergen extract related toxicologically changes were shown in long-time repeated administration of JCP-allergen extract in rats. NOAEL was greater than 10,000 JAU/kg/day for systemic toxicity, equivalent to 300-fold the human dose. In **Chapter IV**, the JCP-allergen extract induced no local irritative response in oral mucosa as well as mucosa of GI tract in rabbit and rats respectively, suggesting that SLIT-swallow at clinical doses should not exert remarkable adverse effects in patients with JC pollinosis. These toxicological studies were conducted using normal animals because the purpose of these studies was to assess the physical properties of the drug. Further study is required in JCP extract -sensitized animals to evaluate the toxicity that appeared during SLIT in patients with JC pollinosis. In **Chapter V**, it was

demonstrated to be no potential in genotoxicity of the JCP-allergen extract and 1,500-fold of safety margin.

Okamoto et al. demonstrated improvement of the nasal and ocular symptoms of JC pollinosis by clinical trial with standardized JCP-allergen extract (manuscript in preparation). Based on present nonclinical studies, the safety profile supports the safe use of standardized JCP-allergen extract for SLIT in JC pollinosis patients.

On the other hand, the mechanism of SLIT is not understood entirely. The changes on immunoglobulin-based immunological reaction (IgG subtype, IgE, IgA in SLIT) and cell-based immune response induced by repeated administration of allergen extract are key events in mechanism of AIT and need to be analyzed.

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